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(71) Applicant *(for all designated States except US)*: SYNCHEM RESERACH, INC. [CA/CA]; 21 Slade Crescent, Kanata, Ontario K2K 2L1 (CA).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: MATHESON, Ronald, C. [CA/CA]; 21 Slade Crescent, Kanata, Ontario K2K 2L1 (CA).

(74) Agents: HICKS, Andrew, R. et al.; Borden Elliot Scott & Aylen, 1000–60 Queen Street, Ottawa, Ontario K1P 5Y7 (CA).

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(54) Title: BIOMIMETIC CHELATING AGENTS AND METHODS

(57) Abstract

A method of controlling systemic copper levels in mammals includes administering to a mammal a compound which is preferentially bound by hepatocytes, serum albumin, or both, and which selectively binds with copper. The compounds can be administered to patients by oral or intravenous or topical routes in a phamaceutically acceptable vehicle, at a dosage which is dependent on the composition of the selected compound on the copper level, body weight of the patient or upon the surface area of skin being treated. The compounds of this invention have particular utility in the treatment of Wilson's Disease, angiogenic forms of cancer, psoriasis, and other pathologies in which angiogenesis causes or exacerbates the disease.

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BIOMIMETIC CHELATING AGENTS AND METHODS

FIELD OF THE INVENTION

This invention relates to methods of *in vivo* chelation of copper in mammals to control accumulated copper levels in tissues undergoing angiogenesis or with toxic levels of copper.

BACKGROUND OF THE INVENTION

Several potentially fatal and/or damaging diseases are known to be either caused or exacerbated by angiogenesis. Angiogenesis is the process of new blood vessel formation in the body. It is a vital process for various physiological functions such as wound healing and menstruation, but localized angiogenesis can also cause or exacerbate diseases such as angiogenic forms of cancer including tumors in the bladder, the brain, the breast, the cervix, the colon, the rectum, the kidney, the lung, the ovary, the pancreas, the prostate, the stomach and the uterus; proliferative retinopathy (the leading cause of blindness in the western world); age-related macular degeneration; rheumatoid arthritis; and psoriasis. (See Folkman (1995) Nat. Med 1:27-31 for review).

Abundant research indicates that both primary and secondary tumors (metastases) require blood vessel proliferation to grow, and the inhibition of blood vessel growth, while not a cure for cancer, can slow, reverse and perhaps even prevent the growth of tumors. Accordingly, treatments which are effective at controlling angiogenesis can be used to retard or prevent cancer growth, providing other anti-tumor therapies a better chance to more effectively destroy the cancerous cells. Potentiation of cytotoxic chemotherapy and of radiotherapy with the concomitant use of angiogenesis inhibitors has been demonstrated (Teicher et al. (1994) Int. J Cancer 57:920-925 and Teicher et al. (1995) Rad Oncol Invest. 2:269-276).

Copper plays a major biochemical role in angiogenesis. In particular, copper is involved in the activation of growth factors such as the dimerization of b-FGF, and serum Cu^{2+}-GHK, activation of angiogenic factors such as Cu^{2+}-(K)GHK derived from SPARC, cross linking of the transitional matrix (e.g., collagens VIII and I by Cu^{2+}-dependent lysyl oxidase), and formation of basement membrane (e.g., collagens IV and elastin by Cu^{2+}-dependent lysyl oxidase).

Biological observations which confirm the important role of copper in angiogenesis have been recently reviewed (see Gullino (1986) Anticancer Res 6:153-158) and findings include that Cu accumulates in tissues prior to vascularization, Cu-deficient animals are unable to mount an angiogenic response to stimulus, resistance to tumor growth shown in Cu-deficient animals, and the ability of Cu complexes to affect biosynthesis of fibronectin and collagen by endothelial cells *in vitro*. Cu complexes have also been found to affect endothelial cell migration *in vitro*, stimulate cord formation *in vitro*, and to act as potent angiogenesis effectors in the cornea.

Angiogenesis is a highly complex process, and many different approaches are being researched to control various stages of the process. However, there is considerable scientific evidence to date which points to targeted copper chelation therapy as a viable approach to angiosuppression. This evidence includes slowing tumor growth in animal models of disease when systemic copper levels are lowered (Brem et al. (1990) Am J. Path. 137:1121-1142). Additionally, investigators have determined that increased serum copper levels, ranging from 50 to 250% above normal, have been observed in a wide variety of cancers. Copper levels generally correlate with the tumor burden and, in remission, copper levels tend to normalize (Linder (1983) J. Nutrition, Growth, Cancer 1:27-38).

Presently, copper chelation therapy is not a viable treatment option, largely because existing agents have sub-optimal copper affinity and selectivity, poor targeting (not tumor specific) and unacceptable patient side effects. Poor targeting is a problem, since angiogenesis is required to support normal development and wound healing. In particular, the inventor is not aware of any known pharmaceutical compositions which employ copper chelation for angiosuppression. Instead, efforts to control angiogenesis have focused on anti-mitotic drugs, metabolites of cortisone, monoclonal antibodies to angiogenic factors and protease inhibitors acting on collagenase (Folkman (1996) Sci. Am. 275:150-154).

Another serious disease in which Cu plays an important role is Wilson's disease. Wilson's disease is an autosomal recessive abnormality in the hepatic excretion of copper that results in toxic accumulations of the metal in the liver, brain, and other organs. The toxic effects of copper in the liver may result in acute hepatitis, fulminant hepatitis, chronic active hepatitis or cirrhosis. The primary neurologic manifestations are those of movement disorder, particularly resting and intention tremors. Spasticity,

rigidity, chorea, drooling, dysphagia, and dysarthria are common. Psychiatric disturbances, primarily due to the toxic effects of copper on the brain, but in some degree reactions to a life-threatening disease, are present in most patients with Wilson's disease. Improvement in the psychiatric state can occur with pharmacologic reduction of the copper excess.

The metabolic defect in Wilson's disease is an inability to maintain a proper balance of copper. Excess copper, small amounts of which are essential to life, accumulate, particularly in the liver. The capacity of hepatocytes to store copper is eventually exceeded and release into blood and uptake in extrahepatic sites occurs.

Under normal circumstances, essentially all tissue copper is present as the prosthetic element of copper proteins, including metallothionein, cytochrome c, oxidase, tyrosinase, superoxide dismutase, and ceruloplasmin. Normally there is little or no free (non-protein-bound) copper. In Wilson's disease more copper is present than can be bound by specific copper proteins. The pathologic consequences of the accumulated copper occur first in the liver. Abnormal fat and glycogen deposits, and mitochondrial abnormalities can be observed early on. Later, necrosis, inflammation, fibrosis, bile duct proliferation, and cirrhosis occur. Death can occur from the effects of copper toxicosis in the central nervous system. Necrosis of neurons with cavitations may be preceded by the appearance of Opalski and Alzheimer type II cells.

Treatment of Wilson's disease consists of removing and detoxifying the deposits of copper as rapidly as possible and should be instituted once the disease has been properly diagnosed, whether the patient is ill or asymptomatic. Penicillamine is currently preferred for the treatment of Wilson's disease. Sensitivity to penicillamine usually appears within the first 14 days of treatment and may cause rash, fever, leukopenia, thrombocytopenia, lymphadenopathy, or proteinuria. Discontinuation of treatment is required if sensitivity develops. Therapy can often be resumed if the drug is re-instituted in small and gradually increasing dosages, although reactions are less likely to recur if prednisone is co-administered with penicillamine. Reactions requiring a desensitizing regimen may recur several times before penicillamine can be administered without a steroid.

Life-long and continual treatment is required. Discontinuation of treatment usually results in death within an average time of 2 to 3 years. Re-institution of penicillamine after temporary interruption of therapy may be accompanied by the

appearance or reappearance of sensitivity reactions. At any time, granulocytopenia (or agranulocytosis), thrombocytopenia, nephrotic syndrome, Goodpasture's syndrome, systemic lupus erythematosus, severe arthralgias, or myasthenia gravis may supervene. Toxicity is sometimes dose-related and reduction of the dose to a level that is therapeuticly effective but non-toxic may be possible. Continued low dosage of glucocorticoids may control penicillamine-associated lupus or arthralgias.

In addition to the undesirable side effects frequently associated with penicillamine, and the potential for permanent intolerance, penicillamine is not highly selective for copper. Penicillamine binds with zinc as well and may create a zinc imbalance. Accordingly, penicillamine treatment is often supplemented with zinc. Also, because of penicillamine's lack of specificity for copper, relatively high doses (such as about 2 grams per day), which can cause toxicity, must often be administered.

Irreversible intolerance to penicillamine can occur. In such cases, penicillamine treatment is discontinued and replaced by trientine, an orphan drug approved by the Food and Drug Administration in 1985.

Because patients with Wilson's disease generally die shortly after treatment is discontinued, patients who develop a permanent intolerance for penicillamine are generally treated with trientine. As with penicillamine, the dose of trientine is generally selected to minimize toxic effects while providing sufficient amounts to effectively treat Wilson's disease.

Another treatment which has been attempted to control systemic copper to help control Wilson's disease, cancer, and other angiogenesis-related diseases is to limit dietary intake of copper. However, this is extremely difficult because many foods, including foods which can be important to maintaining proper nutrition, contain high levels of copper. Additionally, people with cancer or other diseases associated with uncontrolled angiogenesis absorb any available copper at about double that of normal people. Consequently, regulation of the dietary intake of copper is generally ineffective in the treatment of Wilson's disease, cancer, and other diseases associated with uncontrolled, localized angiogenesis, such as proliferative retinopathy, rheumatoid arthritis and psoriasis.

Accordingly, there remains a need for alternative methods of treatment, and pharmaceutically safe compositions, for reducing systemic copper levels in mammals, particularly people with diseases such as Wilson's disease, angiogenic forms of cancer,

and other diseases in which angiogenesis causes or exacerbates the disease. In particular, there remains an unfulfilled need for methods and pharmaceutical compositions which control systemic copper levels and which are biologically stable, chemically stable, non-toxic at effective doses and exhibit a dose-dependent effect.

SUMMARY OF THE INVENTION

This invention is directed to methods of and compounds for controlling systemic copper levels in mammals by administering to mammals a compound which is preferentially bound by hepatocytes, serum albumin, or both, and which selectively binds with copper. The invention has particular utility in the treatment of Wilson's disease, angiogenic forms of cancer, and other diseases in which angiogenesis causes or exacerbates the disease.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a series of three photographs showing arterial density in 9 day embryonic quail CAM after 48 hours of exposure to a control (FIG. 1A), compound 27 at 0.2 mM concentration (FIG. 1B) and compound 27 at 0.5 mM concentration (FIG. 1C);

FIG. 2 is a series of three photographs of arterial density in 8 day embryonic quail CAM after 24 hours of exposure to a control (FIG. 2A), compound 27 at 0.5 mM concentration (FIG. 2B) and compound 27 at 1 mM concentration (FIG. 2C);

FIG. 3 is a series of three photographs showing arterial density in embryonic quail CAM after exposure for 24 hours to a control (FIG. 3A), compound 21 at 0.2 mM (FIG. 3B) and compound 21 at 0.3 mM (FIG. 3C);

FIG. 4 is a series of three photographs of arterial vessel density in embryonic quail CAM after 24 hours of exposure to a control (FIG. 4A), compound 21 at 0.1 mM concentration (FIG. 4B) and compound 21 at 2 mM concentration (FIG. 4C);

FIG. 5A is a graph of arterial branch points per field in 8 day embryonic quail CAM after 24 hours of exposure to a PBS control, compound 27 at 0.5 mM and compound 27 at 1 mM;

FIG. 5B is a graph of primary, secondary and tertiary branch points as a percent of total branching after exposure of 8 day embryonic quail CAM cells for 24 hours by a PBS control, compound 27 at 0.5 mM concentration and compound 27 at 1 mM concentration;

FIG. 6A is a graph showing primary, secondary and tertiary branch points as a percent of total for a 9 day embryonic quail CAM treated for 48 hours (2 applications) with a PBS control, compound 27 at 0.2 mM, compound 27 at 0.5 mM and compound 27 at 1 mM;

FIG. 6B is a graph of tertiary branch points as a percent of total for the same specimens as referenced in FIG. 6A above;

FIG. 7 is a graph of the percent of total tertiary branch points in 8 day embryonic quail CAM treated for 24 hours and 9 day embryonic quail CAM treated for 48 hours in a PBS control, compound 27 at 0.5 mM and compound 27 at 1 mM;

FIG. 8 is a chart of tumor weight at 21 days for mouse melanoma tumors treated with a control, compound 21 at 150 micrograms per day, compound 21 at 300 micrograms per day, compound 22 at 150 micrograms per day, compound 22 at 300 micrograms per day, compound 27 at 150 micrograms per day and compound 27 at 300 micrograms per day;

FIG. 9 is a graph of the same tumors treated in the same way as in FIG. 8, but showing tumor volume at 21 days;

FIG. 10A is a graph of mouse melanoma tumor volume over time, when treated with a control and with compound 21 at 150 micrograms per day;

FIG. 10B is a graph of mouse melanoma tumor volume over time, when treated with a control and with compound 21 at 300 micrograms per day;

FIG. 11A is a graph of mouse melanoma tumor volume over time, when treated with a control and with compound 22 at 150 micrograms per day;

FIG. 11B is a graph of mouse melanoma tumor volume over time, when treated with a control and with compound 22 at 300 micrograms per day;

FIG. 12A is a graph of mouse melanoma tumor volume over time, when treated with a control and with compound 27 at 150 micrograms per day;

FIG. 12B is a graph of mouse melanoma tumor volume over time, when treated with a control and with compound 27 at 300 micrograms per day;

FIG. 13A, B and C show the frequency distribution of human prostate tumor volume (in mice), as between those smaller than 300 cubic millimeters and those larger than 300 cubic millimeters, where the subjects have been treated with a control (FIG. 13A), compound 27 at 250 micrograms per day (FIG. 13B) and compound 27 at 500 micrograms per day (FIG. 13C);

FIG. 14A, B and C show the frequency distribution of human prostate tumor weight (in mice), as between those smaller than 300 milligrams and those larger than 300 milligrams, where the subjects have been treated with a control (FIG. 14A), compound 27 at 250 micrograms per day (FIG. 14B) and compound 27 at 500 micrograms per day (FIG. 14C);

FIG. 15 is a graph of human prostate tumor volume over time, comparing subjects treated with control to subjects treated with compound 27 at 500 micrograms per day;

FIG. 16 is a graph of human prostate tumor volume over time, comparing subjects treated with control to subjects treated with compound 27 at 250 micrograms per day;

FIG. 17 is regression analysis charting final tumor volume vs. serum copper for subjects treated with compound 22 at 500 micrograms per day, compound 27 at 250 micrograms per day and a control; and

FIG. 18 is regression analysis charting final tumor volume against serum zinc for the three treatments discussed in FIG. 17 above, combined.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

THE COMPOUNDS OF THE INVENTION

The compounds which are useful in the practice of this invention are those which are sufficiently lipophilic to be preferentially conjugated, bound or taken up by normally functioning hepatocytes as compared with other tissues, or to serum albumin as compared with other plasma proteins, or both, and which selectively bind to copper as compared with other bioavailable metal ions. Compounds having sufficient lipophilicity are those which have a non-polar structure or which have a low polar structure. Suitable compounds generally include those having lipophilic groups such as an alkyl or aryl group (e.g., a substituted or unsubstituted phenyl ring). Compounds which exhibit suitable lipophilicity for use in practicing the invention generally do not carry an ionic charge greater than 3 at neutral pH. The compounds used in the practice of this invention preferably have a binding constant of at least 10^{15} for copper.

To facilitate absorption and uptake, the compounds used in the method of this invention should exhibit moderate water solubility, and preferably should be soluble to a concentration of at least 0.1 mmol per liter in a normal saline solution.

The compounds used in the practice of this invention should have an acceptably low toxicity level at the dosages used. The toxicity levels of the compounds should generally be at least 0.001 mmol per kilogram of body weight of the mammal being tested, and more preferably at least 0.01 mmol per kilogram of the body weight of the mammal being tested. Toxicity levels in this specification refer to the administered dose at which 50% of a test population dies, referred to as LD50.

The word "mammal" as used herein refers to all mammals, including humans.

The compounds used in the practice of this invention should have a molecular weight of at least 250, and more preferably at least 300, to maximize binding to HSA and/or uptake by the ultimate *in vivo* target. The molecular weight, however, must not be so high as to prevent or inhibit systemic absorption when taken orally or applied topically.

For treating the liver, the biomimetic compound is selected to be more easily extracted by the liver from the blood. For treating tumors, the biomimetic compound is selected to be retained by albumin and to minimize uptake by the hepatocytes. For treating skin, the biomimetic compound is selected to be more easily absorbed through the skin and to be highly stable to air and light.

The compounds which are useful in the practice of this invention are polydentate ligands that have a binding constant of at least 10^{15} for Cu(II), preferentially bind Cu(II) as compared with other first row transition metal ions such as Zn(II), Ni(II) etc., and preferably have lipophilic character. It is believed without wishing to be bound by such belief, that lipophilic character will facilitate the absorption of these polydentate ligands from the gut and also enhance their uptake by serum albumin and/or hepatocytes. According to the present invention there are provided polydentate ligands corresponding to the formula:

$$N_x(R)_B \; aH_cY$$

wherein Nx refers to a linear, macrocyclic, or caged (strapped macrocyclic) polydentate amine ligand containing up to 60 nonhydrogen atoms, in which x is the number of nitrogen donor groups N, and is a number from 3 to 12, and the nitrogen-donor groups are separated by 2-3 nonhydrogen atoms so that metal binding results in linked 5- and 6-membered chelate rings; HcY is an acid that may be used to partially or fully neutralize the amine groups; C is a number from 1 to 3; a is the number, from 0 to 12, of moles of acid used to convert the polydentate amine to a salt; R is a lipophilic moiety such as an

donor ligands themselves and can enhance the binding of metal ions such as Cu(II); B is the number of lipophilic moieties R present and is a number from 0 to 12; and R may be attached to a nitrogen donor atom or a nonhydrogen atom.

Additionally, according to the present invention there are provided processes for preparing such polydentate ligands and their salts.

The present invention also provides for preloading the polydentate ligand with a positive ion such as Zn(II) that may be displaced by Cu(II); such metal complexes will have anionic counter-ions (Y) for charge balance;

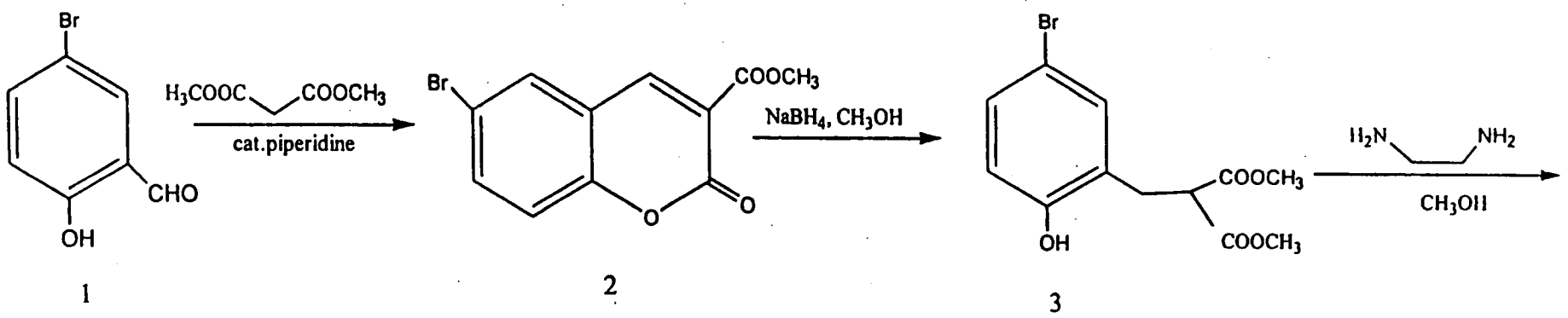
The polydentate ligands described in the present invention are novel and desirable agents that target copper chelation and achieve control of angiogenesis-linked pathologies. Assays and experiments discussed herein demonstrate the low toxicity of such polydentate ligands as well as their usefulness for controlling angiogenesis and tumor growth rates.

SYNTHESIS OF EXEMPLARY COMPOUNDS

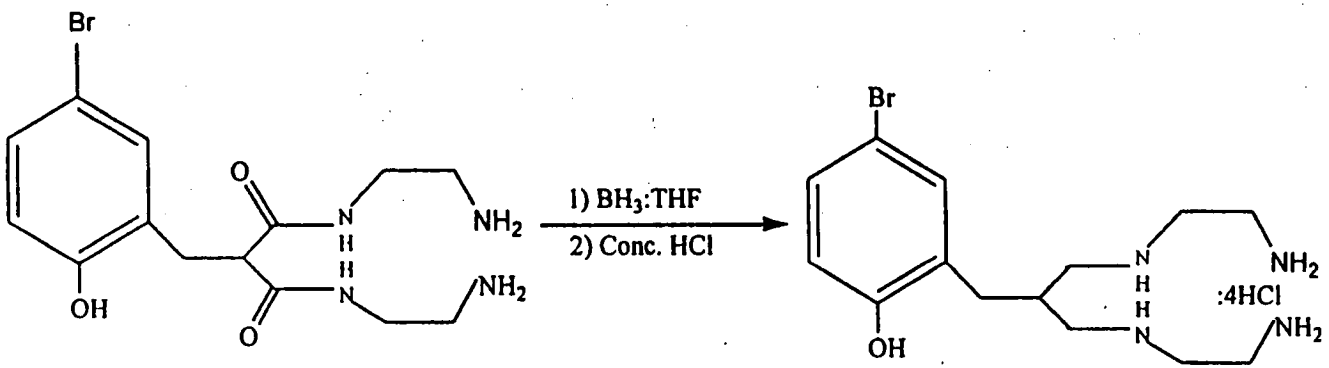
The compounds represented by formula I can be synthesized by procedures known in the art. Specific examples of compounds represented by formula I (compounds 5, 13, 15, 21, 22 and 27) and appropriate intermediary and control compounds can be synthesized by Schemes 1-7 which follow:

POLYAMINE LIGANDS

Scheme-1

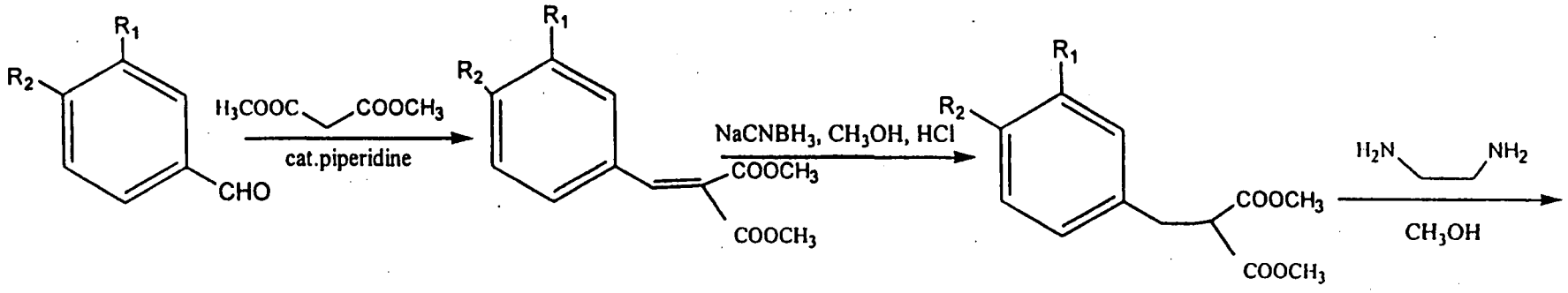


1 2 3



4 5

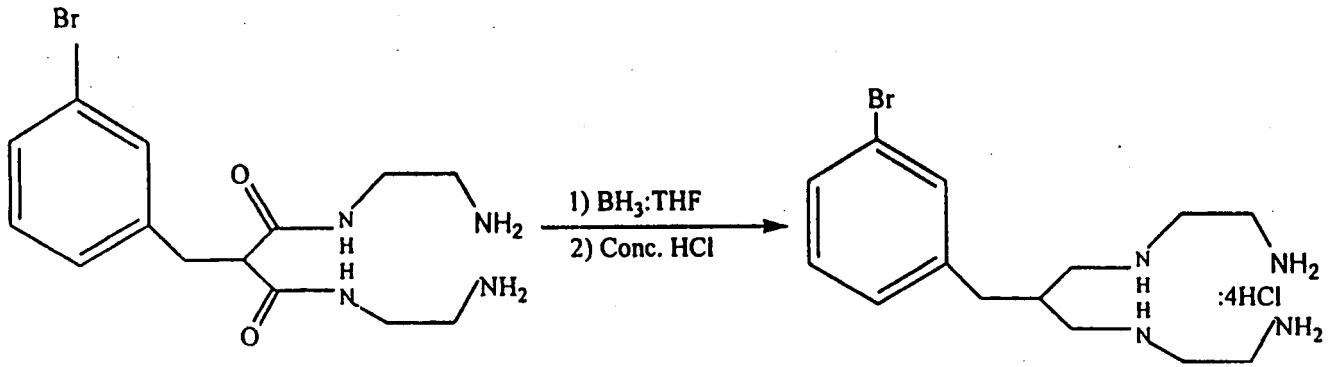
Scheme-2



6: R_1 = Br, R_2 = H
7: R_1 = H, R_2= Isopropyl

8: R_1 = Br, R_2 = H
9: R_1 = H, R_2= Isopropyl

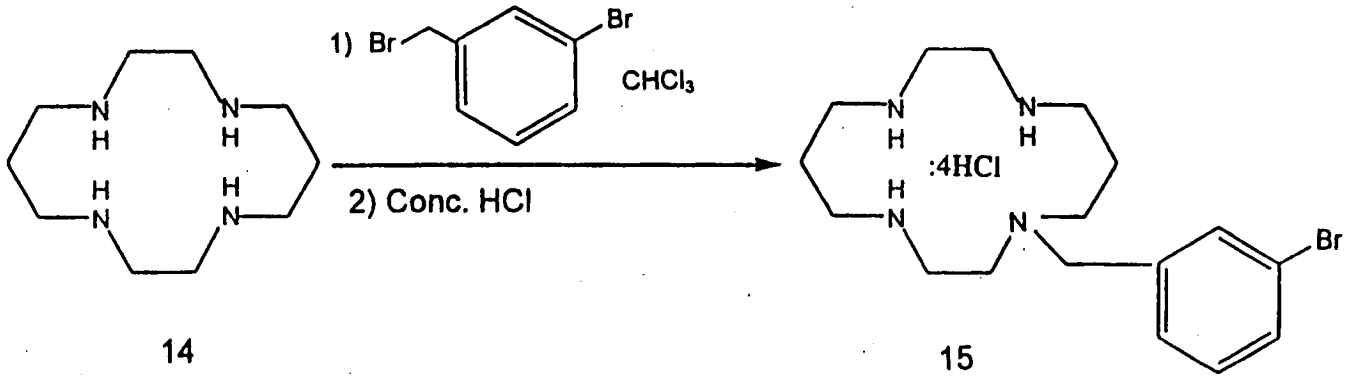
10: R_1= Br, R_2= H
11: R_1 = H, R_2 = Isopropyl



12

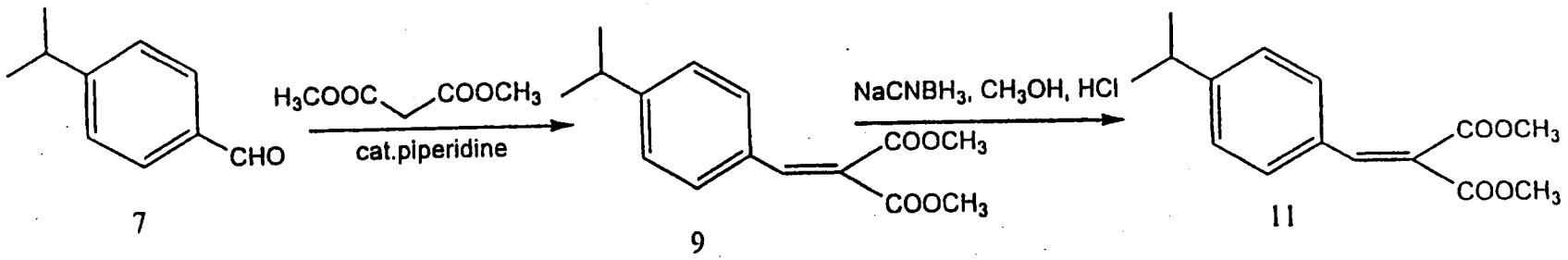
13

Scheme-3



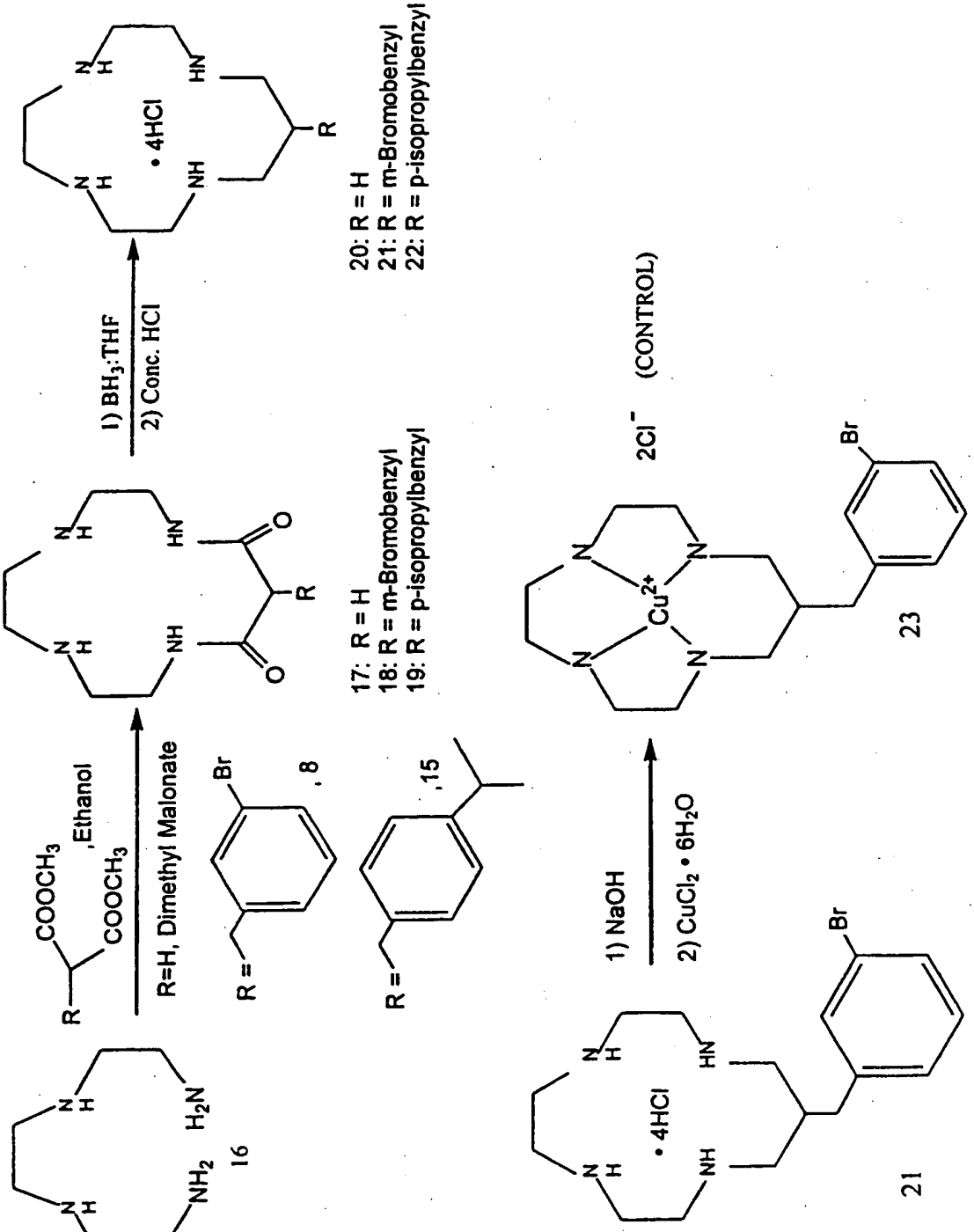
14 15

Scheme-4

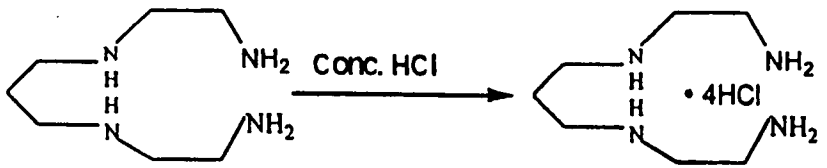


7 9 11

Scheme-5

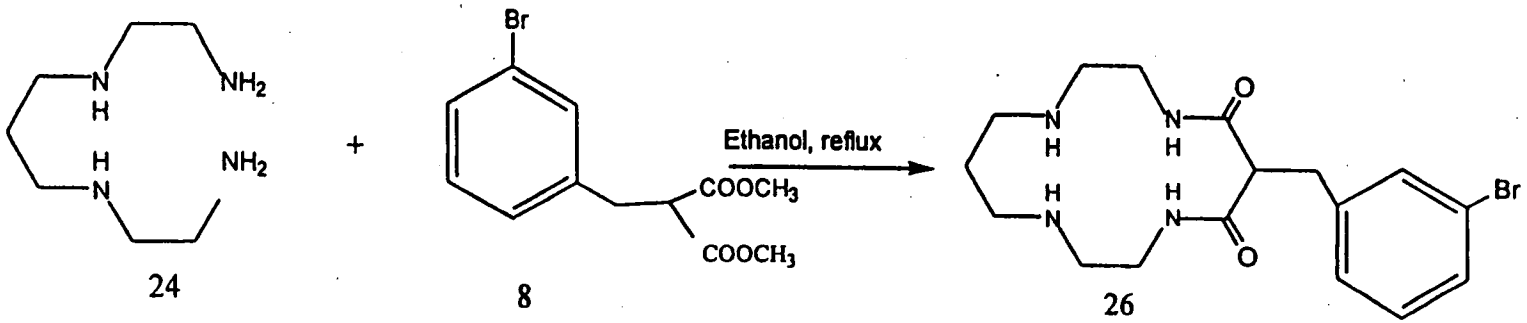

16
COOCH3
COOCH3
R
,Ethanol
R=H, Dimethyl Malonate
R =
,8
,15
17: R = H
18: R = m-Bromobenzyl
19: R = p-isopropylbenzyl
1) BH3:THF
2) Conc. HCl
• 4HCl
20: R = H
21: R = m-Bromobenzyl
22: R = p-isopropylbenzyl
1) NaOH
2) CuCl2 • 6H2O
2Cl-
(CONTROL)
Cu2+
23
21

Scheme-6

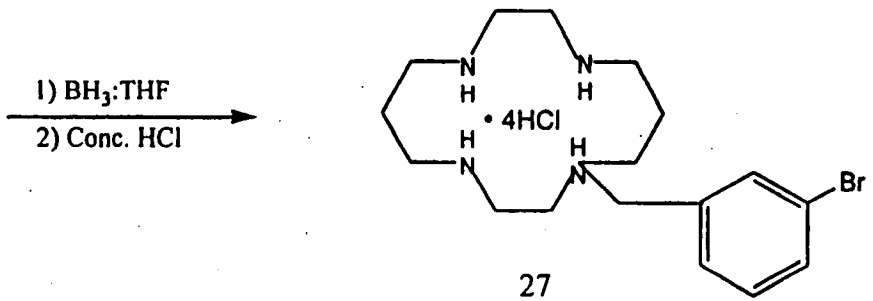


24 25

Scheme-7



24 8 26



27

The lipophilic polyamine salts which have been synthesized include the linear molecules 6-(3-bromo-6-hydroxyphenyl)methyl-1,4,8,11-tetraazaundecane tetrahydrochloride (5) and 6-(3)bromophenyl)methyl-1,4,8,11-tetraazaundecane tetrahydrochloride (13), and the macrocyclic molecules 1-(3-bromophenyl)methyl-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride (15), 6-(3-bromobenzyl)-1,4,8,11-tetraazacyclotridecane tetrahydrochloride (21), 6-(4-isopropylbenzyl)-1,4,8,11-tetraazacyclotridecane tetrahydrochloride (22), and 6-(3-bromobenzyl)-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride (27). As will be readily recognized by workers in this field, many lipophilic polyamine analogs can be prepared for which the number and separation of nitrogen-donor groups may be varied, the point(s) of attachment of the lipophilic group(s) may be varied, the lipophilic moiety may include linear, branched, or cyclic aliphatic groups, heterocyclic groups, and other aromatic groups. Salts of polyamines can be prepared with acids other than hydrochloric acid. Examples of polyamine analogs embodied within Schemes 1-7 include variations in size of the macrocycle ring (21 and 27), variations in the point of attachment of the lipophilic group (15 and 27), and variations in the lipophilic group (5, 22 and 27).

Owing to the relatively large affinity of Cu(II) for nitrogen-donor ligands, it is well known that linear and macrocyclic triamine or higher polyamine chelating agents show binding constants for Cu(II) that exceed 10^{15} (*M. Kodama et al., J.C.S. Dalton*, pp. 1081-85 (1978)). The strong binding of Cu(II) to macrocyclic polyamines having as many as twelve nitrogen-donor ligands has been reported (*A. Bianchi et al, Pure & Appl. Chem. 60*, pp. 525-32 (1988)). Additionally, chelating agents can be constructed whereby two macrocyclic polyamine chelating agents are linked by chains of nonhydrogen atoms (*E. Kimura et al., J. Org. Chem. 55*, pp. 42-46 (1990)). Moreover, the lipophilic groups themselves may have ligand donor atoms such as phenols, thiols, pyrazoles, imidazoles, and carboxylic acids that can also bind Cu(II) (*E. Kimura et al., Inorg. Chem. 29*, pp. 4991-96 (1990); *W.J. Kruper et al., J. Org. Chem. 58*, pp. 3869-76 (1993)). Complexes of this type are said to have such "pendent coordinating groups". Additionally, chelating agents that can "cage" a metal ion are made by strapping a macrocyclic ligand with a pendent group (*P.V. Bernhardt et al., Coord. Chem. Reviews 104*, pp. 297-343 (1990)).

Workers in this field will also appreciate that lipophilic polyamine ligands may be prepared by a variety of synthetic routes (*Macrocycle Synthesis, D. Parker, Ed., Oxford University Press, New York*, 1996, pp. 1-23)).

EXAMPLE 1 6-(3-Bromo-6-hydroxyphenyl)methyl-1,4,8,11-tetraazaundecane tetrahydrochloride (5)

As outlined in Scheme 1, a mixture of 5-bromosalicylaldehyde (1) (*Aldrich Chemical Co.*, 5.0 g, 25 mmol) and dimethyl malonate (*Aldrich Chemical Co.*, 3.8 g, 29 mmol) was gently warmed (for homogeneity) and piperidine (6-8 drops) was added. The resulting yellow reaction mixture was tightly stoppered and left at room temperature overnight. The solid was triturated with benzene (50 mL), filtered, washed with benzene (3x10 mL) and dried to yield methyl-6-bromo-coumarin-3-carboxylate (2) as a fluffy off-white solid (6.1 g, 86%): m.p. 165-168°C open capillary, clear liquid; ^{1}H NMR (200 MHz, $CDCl_3$) d 3.97 (s, -COOC$\underline{H}_3$), 7.26 (dd, J = 0.74, 9.88, H-8), 7.70-7.76 (m, H-7), 7.75 (s, H-5), 8.48 (s, H-4).

To a cooled (0°C) solution of (2) (6.0 g, 21.2 mmol) in anhydrous methanol (60 mL), sodium borohydride (*Aldrich Chemical Co.*, 1.05 g, 27.6 mmol) was added in four portions. After 0.5 h, when the effervescence had ceased, methanol was distilled off in vacuo and the residue was taken up in water (100 mL) and extracted with ethyl acetate (2x125 mL). The combined organic extracts were dried over sodium sulfate and concentrated in vacuo to yield dimethyl-α-(3-bromo-6-hydroxyphenyl)methylmalonate (3) as an oily residue which could be used as such (6.1 g, 91%). An analytical sample was purified by flash column chromatography on 230-400 mesh silica by using dichloromethane as the eluent: ^{1}H NMR (200 MHz, $CDCl_3$) d 3.14 (d, J = 7.3, benzylic-C$\underline{H}_2$), 3.75 (s, -CH(COOC$\underline{H}_3$)2), 3.80 (m, -C$\underline{H}$(COOCH$_3$)2), 6.73 (d, J = 10.0, ar-H-5), 7.19-7.30 (m, ar-H-2,4).

To a solution of (3) (3.9 g, 12.3 mmol) in anhydrous methanol (18 mL), ethylene diamine (*Aldrich Chemical Co.*, 8.3 mL, 123 mmol) was added and the resulting clear solution was stirred at room temperature for 96 h. The volatiles were then distilled off in vacuo and the gummy residue was dried under vacuum for 24 h to yield 6-(3-bromo-6-hydroxyphenyl)methyl-5,7-dioxo-1,4,8,11-tetraazaundecane (4) as a pale yellow solid (4.6 g, 100%). An analytical sample was washed with dichloromethane and dried: ^{1}H NMR (200 MHz, DMSO-d$_6$) d 2.92 (d, J = 6.96, benzylic-C$\underline{H}_2$), 2.94-3.13 (m, H-

2,10), 3.37-3.70 (m, H-3, 6, 9), 6.73 (d, J = 8.72, ar-H-5), 7.14 (s, ar-H-2), 7.15 (d, J = 6.3, ar-H-4), 7.92 (br m, amide NH's).

To the solid (4) (2.0 g, 5.1 mmol) under argon, a 1 M solution of borane-tetrahydrofuran complex in tetrahydrofuran (*Aldrich Chemical Co.*, 60 mL) was added slowly and vigorous hydrogen evolution was observed. The clear solution was then refluxed for 50 h under argon. The excess reagent was quenched by cautiously adding methanol (10 mL). the volatiles were then distilled off in vacuo, 40 mL of concentrated HCl was added to the residue followed by extraction with chloroform (3x50 mL). The aqueous solution was basified with sodium hydroxide and extracted with chloroform (3x150 mL). The chloroform extracts were dried over sodium sulfate and concentrated in vacuo to afford 6-(3-bromo-6-hydroxyphenyl)methyl-1,4,8,11-tetraazaundecane as a yellow oil (0.8 g, 45%). The amine (0.17 g) was dissolved in ethanol (3 mL), concentrated HCl (3 mL) was added and the volatiles were distilled off in vacuo to yield the salt (5) as a pale yellow hygroscopic solid foam (0.19 g): ^{1}H NMR (200 MHz, D_2O) d 2.46-2.67 (m, H-6), 2.76 (d, J = 6.4, benzylic-CH_2), 3.13 (t, J = 6.2, H-5,7), 3.33 (s, H-2,3,9,10), 6.78 (d, J = 9.2, ar-H-5), 7.20-7.34 (m, ar-H-2,4); ^{13}C NMR (50 MHz, D_2O) d 32.08, 37.19, 38.23, 47.86, 51.86, 114.73, 120.27, 127.99, 134.49, 136.70, 156.41.

EXAMPLE 2 6-(3-Bromophenyl)methyl-1,4,8,11-tetraazaundecane tetrahydrochloride (13)

As outlined in Scheme 2, a few drops of piperidine were added to a mixture of 3-bromobenzaldehyde (6) (Aldrich Chemical Co., 4.62 g, 25 mmol) and dimethylmalonate (3.8 g, 29 mmol), and the resulting clear solution was warmed to 40°C for 24 h.[1] The turbid reaction mixture was dissolved in 25 mL of ethanol and 30 mL of water and heated to boiling. The clear solution was slowly cooled to room temperature. White needles of dimethyl-2-(3-bromophenyl)-1, 1-ethylenedicarboxylate (8) were collected by filtration and dried in air (2.01 g, 43%): m.p.54-56°C, clear liquid, open capillary; ^{1}H NMR (200 MHz, $CDCl_3$) d 3.86 (s, -CH($COOCH_3$)$_2$), 7.20-7.39 (m, ar-H-5,6), 7.50-7.61 (m, ar-H-2,4), 7.69 (s, olefinic-H).

To a cooled (0°C) solution of (8) (3.01 g, 10 mmol) in anhydrous methanol (44 mL) under argon, sodium cyanoborohydride (0.75 g, 12 mmol) was added in 3 portions. The pH of the reaction mixture was then adjusted to 4 by adding conc. HCl dropwise every 0.5 h till the solution attained a pale yellow colour (at pH = 4 the solution turns

pale yellow). The reaction was stirred overnight at room temperature and subsequently poured into water (300 mL) and extracted into dichloromethane (2x300 mL). The combined organic extracts were dried over sodium sulfate and concentrated in vacuo to afford dimethyl-α-(3-bromophenyl)methylmalonate (10) as a colourless oil which solidified on standing (2.9 g, 97%): ^{1}H NMR (200 MHz, $CDCl_3$) d 3.19 (d, J =7.8, benzylic-CH_2), 3.65 (t, J = 8.0, -C<u>H</u>($COOCH_3$)$_2$), 7.13-7.16 (m, ar-H-5,6), 7.34-7.37 (m,ar-H-2,4).

To a solution of (10) (3.0 g, 10 mmol) in anhydrous methanol (12 mL) under argon ethylene diamine (6.6 mL, 100 mmol) was added and the resulting clear solution was stirred at room temperature for 96 h during which time a solid was formed. The volatiles were distilled off in vacuo, and 6-(3-bromophenyl)methyl-5,7-dioxo-1,4,8,11-tetraazaundecane (12) was isolated as a light yellow solid and dried under vacuum for 24 h (3.5 g, 100%): ^{1}H NMR (200 MHz, DMSO-D_6) d 2.85-3.13 (m, H-2, 10, benzylic-CH_2), 3.20-3.41 (m, H- 3,6,9), 7.20 (m, ar-H-5,6), 7.40 (m ar-H-2,4), 7.90-8.21 (br m, amide NH's).

A solution of (12) (1.4 g, 4 mmol) in tetrahydrofuran was reduced with (28 mL) of a 1<u>M</u> borane:THF solution by refluxing for 50h using the procedure described above for (5). 6-(3-Bromophenyl)methyl-1,4,8,11-tetraazaundecane was isolated as its pale yellow hygroscopic tetrahydrochloride salt (13) (0.7 g, 53%): ^{1}H NMR (200 MHz, D_2O) d 2.43-2.62 (m, H-6), 2.77 (d, J = 7.4, benzylic-CH_2), 3.05(dd, J = 6.2,13.2,H-5,7), 3.24(dd, J = 6.6, 3.0, H-5,7), 3.40 (s, H-2,3,9,10), 7.12-7.30 (m, ar-H-5,6), 7.39-7.51 (ar-H-2,4); ^{13}C NMR (50 MHz, D_2O) d 37.73, 38.01, 38.24, 38.63, 47.97, 51.82, 125.23, 130.95, 133.30, 133.66, 134.88, 141.86.

EXAMPLE 3 <u>1-(3-Bromophenyl)methyl-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride (15)</u>

As outlined in Scheme 3, a solution of 1,4,8,11-tetraazacyclotetradecane (14) (Aldrich Co., 0.25 g, 1.25 mmol) and 3-bromobenzylbromide (Aldrich Co., 0.45 g, 1.88 mmol) in chloroform (32 mL) was stirred at room temperature under argon for 30 h.[3] The reaction mixture was applied to a 230-400 mesh silica column using 16:3:0.5 chloroform : methanol : conc. ammonium hydroxide as the eluent. The free base of the monoalkylated product (15) was eluted last and was obtained as a brownish-yellow oil (0.14 g, 30.3%). The oil was dissolved in conc. HCl (3 mL), the solution was heated and 22 mL of ethanol was added. After cooling to room temperature the white

precipitate that formed was filtered, washed with ethanol and dried to yield 1-(3-bromophenyl)methyl-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride (15) as an off-white nonhygroscopic solid (0.12 g): ^{1}H NMR (200 MHz, D_2O) d 2.06 (m, H-6, 13), 3.07 (t, 2H), 3.11-3.30 (m, 8H), 3.30-3.53 (m, 6H), 4.08 (s, benzylic-CH_2), 7.35 (m,ar-H-5,6), 7.58 (m, ar-H-2,4); ^{13}C NMR (50 MHz, D_2O) d 19.65, 21.49, 22.00, 40.88, 41.17, 44.40, 44.97, 47.97, 50.95, 60.29, 60.90, 125.50, 132.43, 133.99, 134.65, 136.06, 136.30.

EXAMPLE 4 1,4,7,10-tetraazacyclotridecane tetrahydrochloride (20)

As outlined in Scheme 5, a solution of dimethyl malonate (6.6 g, 50 mmol) and triethylenetetramine hydrate (16) (7.3g, 50 mmol) in ethanol (50 mL) was refluxed under argon for 72 h.[4] Ethanol was distilled off in vacuo and the gummy residue was subjected to flash column chromatography on 230-400 mesh silica gel using 10% methanol in chloroform followed by 8:4:0.5 chloroform:methanol:concentrated ammonia as the eluent to afford (17) as a yellowish solid (1.4g 13%): ^{1}H NMR (200 MHz, $CDCl_3$) d 2.78 (s, H-5,6), 2.77-2.86 (m, H-3, 8), 3.07 (s, amine NH's), 3.24 (s, H-12), 3.37-3.46 (m, H-2, 9), 7.89 (s, amide-NH's).

To 11,13-dioxo-1,4,7,10-tetraazacyclotridecane (17) (1.1g, 5.1 mmol) 50 mL of a 1M solution of borane-tetrahydrofuran complex in tetrahydrofuran was added and a vigorous evolution of hydrogen was observed. The resulting solution was refluxed under argon for 36 h and cooled. The reaction mixture was then cooled and the excess reagent was quenched by cautiously adding 5 mL of methanol. The volatiles were distilled off in vacuo and the residue was taken up in 30 mL of 50% HCl and extracted with chloroform (3x30 mL). The aqueous layer was neutralized with sodium hydroxide pellets and extracted with chloroform (3x50 mL). The combined chloroform extracts were dried over sodium sulfate and concentrated in vacuo to a viscous pale yellow oil (0.7 g, 74%). The oil was dissolved in 3 mL of ethanol and 2 mL of concentrated HCl was added. The suspension was heated to boiling and water was added dropwise until the solution just turned clear. After cooling to room temperature, the solution deposited white crystals of (20) which were filtered and dried *in vacuo* to yield 0.6g of 1,4,7,10-tetraazacyclotridecane tetrahydrochloride (**20**): ^{1}H NMR (200 MHz, D_2O) d 2.18 (quintet, J = 6.58, H-12), 3.15 (s, H-5,6), 3.28-3.42 (m, H-2, 3, 8, 9, 11, 13); ^{13}C NMR (50 MHz, D_2O) d 23.60, 45.49, 45.60, 47.07.

EXAMPLE 5 12-(3-Bromophenyl)methyl-1,4,7,10-tetraazacyclotridecane tetrahydrochloride (21)

As outlined in Scheme 5, a solution of dimethyl-α(3bromophenyl)methylmalonate (8, Scheme 2) (12.0 g, 39.8 mmol) and triethylenetetramine hydrate (16) (5.8g, 39.8 mmol) in ethanol (80 mL) was refluxed under argon for 72 h.[4] Ethanol was distilled off in vacuo and the gummy residue was subjected to flash column chromatography on 230-400 mesh silica gel using 200:75:10 dichloromethane:methanol:concentrated ammonia as the eluent to afford 12-(3-bromophenyl)methyl-11,13-dioxo-1,4,7,10-tetraazacyclotridecane (18) as a yellowish solid (2.6g, 17%): ^{1}H NMR (200 MHz, $CDCl_3$) d 2.09 (s, amine NH's), 2.67 (s, H-5, 6), 2.64-2.79 (m, H-3, 8), 3.10-3.35 (m, H-2, 9), 3.17 (d, J = 7.3,benzylic-CH_2), 3.39 3.46-3.50 (m, H-2, 9), 7.13 (d, J = 6.58,ar-H), 7.26 (s, NH's), 7.26-7.38 (m, 3-ar-H's).

To 12-(3-bromophenyl)methyl-11,13-dioxo-1,4,7,10-tetraazacyclotridecane (18) (2.6g, 6.8 mmol) 80 mL of a 1M solution of borane-tetrahydrofuran complex in tetrahydrofuran was added and a vigorous evolution of hydrogen was observed. The resulting solution was refluxed under argon for 96 h and cooled. The reaction mixture was then cooled and the excess reagent was quenched by cautiously adding 5 mL of methanol. The volatiles were distilled off in vacuo and the residue was taken up in 80 mL of 50% HCl and extracted with dichloromethane (2x 100 mL). The aqueous layer was neutralized with sodium hydroxide pellets and extracted with dichloromethane (4x100 mL). The combined dichloromethane extracts were dried over sodium sulfate and concentrated in vacuo to a viscous pale yellow oil (2.0 g, 83%). The oil was dissolved in 30 mL of ethanol and 10 mL of concentrated HCl was added. The precipitated white solid was filtered and taken up in hot ethanol (80 mL) and water was added dropwise to the suspension until it just turned clear. The solution was allowed to cool to room temperature, and white plates that deposited were filtered and dried in air to yield 1.3 g of 12-(3-bromophenyl)methyl-1,4,7,10-tetraazacyclotridecane tetrahydrochloride (21): ^{1}H NMR (200 MHz, D_2O) d 2.30-2.50 (m, H-12), 2.68 (d, J = 7.6, benzylic-CH_2), 3.10 (s, H-5,6), 3.04-3.11 (m, H-3, 8), 3.11-3.18 (m, H-2, 9, 11, 13), 7.19-7.25 (m, 3-ar-H's), 4.43-7.46 (m, 2-ar-H's); ^{13}C NMR (50 MHz, D_2O) d 38.45, 46.61, 47.81, 51.54, 125.19, 130.93, 133.13, 133.59, 134.84, 134.91, 142.72.

EXAMPLE 6 12-(4-isopropylphenyl)methyl-1,4,7,10-tetraazacyc otridecane tetrahydrochloride (22)

As outlined in Scheme 5, a solution of dimethyl-α-(4-isopropylphenyl(methylmalonate (11 Scheme 2) (14.5 g, 54.9 mmol) and triethylenetetramine hydrate (16) (8.03g, 54.9 mmol) in ethanol (110 mL) was refluxed under argon for 72 h.[4] Ethanol was distilled off in vacuo and the gummy residue was subjected to flash column chromatography on 230-400 mesh silica gel using 25% methanol in dichloromethane followed by 30:10:1 dichloromethane:methanol:concentrated ammonia as the eluent to afford 12-(4-isopropylphenyl)methyl-11,13-dioxo-1,4,7,10-tetraazacyclotridecane (19) as a yellowish solid (2.1g, 10.5%): m.p. 203-206°C open capillary with decomposition; ^{1}H NMR (200 MHz, $CDCl_3$) d 1.18 (d, J = 6.9, $CH(\underline{CH_3})_2$), 2.58 (s, H-5, 6), 2.58-2.70 (m, H-3, 8), 2.84 (septet, J = 6.9, $\underline{CH}(CH_3)_2$), 3.15 (d, J = 7.12, benzylic-CH_2), 3.05-3.30 (m, H-2,9), 3.46(appt, J = 7.00,H-12), 4.41-3.60 (m, H-2, 9), 7.10 (s, ar-H-2,3,5,6), 7.45 (s, amide NH's).

To 12-(4-isopropylphenyl)methyl-11,13-dioxo-1,4,7,10-tetraazacyclotridecane (19) (2.0g, 5.78 mmol) 60 mL of a 1 M solution of borane-tetrahydrofuron complex in tetrahydrofuran was added and a vigorous evolution of hydrogen was observed. The resulting solution was refluxed under argon for 96 h and cooled. The reaction mixture was then cooled and the excess reagent was quenched by cautiously adding 5 mL of methanol. The volatiles were distilled off in vacuo and the residue was taken up in 100 mL of 50% HCl and extracted with dichloromethane (3x100 mL). The aqueous layer was neutralized with sodium hydroxide pellets and the oil that separated out was extracted into dichloromethane (4x100 mL). The combined dichloromethane extracts were dried over sodium sulfate and concentrated in vacuo to a viscous pale yellow oil (1.2 g, 65%). The oil was dissolved in 30 mL of ethanol and 10 mL of concentrated HCl was added. The precipitated white solid was filtered and taken up in hot ethanol (50 mL) and water was added dropwise to the suspension until it just turned clear. The solution was allowed to cool to room temperature and deposited white plates. The recrystallization of this product afforded 0.4 g of 12-(4-isopropylphenyl)methyl-1,4,7,10-tetraazacyclotridecane tetrahydrochloride (22): ^{1}H NMR (200 MHz, D_2O) d 1.15 (d, J = 6.96, $CH(\underline{CH_3})_2$), 2.47 (quintet, J = 6.38, H-12), 2.71 (d, J = 7.32, benzylic-CH_2), 2.86 (septet, J = 6.96, $\underline{CH}(CH_3)_2$), 3.15 (s, H-5,6), 3.14-3.18 (m, H-3, 8), 3.22-3.25 (m, H-2,9,11,13), 7.24(ABq, ar-H-2,3,5,6); ^{13}C NMR (50 MHz, D_2O) d

26.10, 36.06, 38.12, 38.26, 46.31, 47.44, 47.95, 50.49, 129.89, 132.16, 137.41, 151.41.

EXAMPLE 7 6-(3-Bromobenzyl)-1,4,8,11-tetraazacyclotetradecanetetrahydrochloride (27)

A solution of dimethyl-α-(3-bromobenzyl)malonate (8, Scheme 2) (9.39 g, 31.2 mmol) in 70 mL methanol was deoxygenated for ten minutes using an argon bubbler, 1,4,8,11-tetraazaundecane (5.0 g, 31.2 mmol) was added, and the colorless solution was refluxed for two days.[4] Methanol was removed by retoevaporation at aspirator pressure, and the oily residue was subjected to flash column chromatography on 230-400 mesh silica gel using a solvent mixture of 25:10:1 methylene chloride:methanol:concentrated aqueous ammonia as the eluent to afford 6-(3-bromobenzyl)-5,7-dioxo-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride (26) as a white solid (1.49 g, 12%): m.p. 192-95°C clear liquid, open capillary; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.52-1.66 (m, H-13), 1.85 (br s, NH's), 2.50-2.79 (m, H-2, 10, 12, 14), 3.09-3.30 (benzylic CH_2), 3.45-3.61 (m, H-3, 9), 7.07-7.18 (m, 2 ar-H's, NH's), 7.29-7.37 (m, 2-ar-H's).

To the solid 6-(3-bromobenzyl)-5,7-dioxo-1,4,8,11-tetraazacyclotetradecane (26) (1.49 g, 3.73 mmol) 67 mL (18 equivalents) of a 1 M solution of borane-tetrahydrofuran complex in tetrahydrofuran was added under an argon atmosphere over a five minute period and the resulting solution was refluxed for 36 hours. The reaction mixture was cooled to room temperature and then was solely quenched with 10 mL of methanol. The volatiles were removed by rotoevaporation under aspirator pressure, and the residue was dissolved in 100 mL of 6 M aqueous HCl. The resulting solution was refluxed for one hour, and cooled to room temperature, and then was extracted with dichloromethane (2x100 mL). This dichloromethane extract was discarded. The aqueous phase was maintained below 25°C (with cooling using an ice bath) during neutralization to pH 12 with sodium hydroxide pellets. The resulting suspension of the product macrocyclictetramine was extracted with dichloromethane (3x100 mL), and the combined organic phases were dried using anhydrous sodium sulfate. The dichloromethane was removed by rotoevaporation under aspirator pressure, and afforded a yellowish white residue. The residue was dissolved in 15 mL of absolute ethanol, and the solution heated to boiling, and 2 mL of concentatred HCl was slowly added. This hot mixture turned cloudy, and after cooling to 0°C, deposited 6-(3-bromobenzyl)-

1,4,8,11-tetraazacyclotetradecanetetrahydrochloride 27 as a white solid that was collected by filtration and dried in air and finally under high vacuum (1.27 g, 80% based on 26). ^{1}H NMR (200 MHz, D_2O) δ 2.01 (quintet, J = 5.60, H-13), 2.28-2.48 (m, H-6), 2.68 (d, J = 7.3, benzylic CH_2), 2.88-3.36 (m, H-2,3,5,7,9,10,12,14), 7.20-7.31 (m, 2 ar-H's), 7.46-7.52 (m, 2 ar-H's); ^{13}C NMR (50 MHz, D_2O) δ 25.74, 38.78, 38.89, 47.32, 47.73, 49.32, 54.82, 124.98, 130.87, 132.73, 133.38, 143.75, 143.46.

REFERENCES

1. *Organic Reactions*, 15, 271.

2. Kirkiacharian, B.S.; Brion, J.-D.; Billet, D. *C.R. Acad. Sc. Paris*. t.294, 1982, Serie II, 181-184.

3. Kruper Jr., W.J.; Rudolf, P.R.; Langhoff, C.A. *J. Org. Chem.*, 58, 1993, 3869-3876.

4. Tabushi, I.; Taniguchi, Y.; Kato, H. *Tet. Lett.*, 12, 1977, 1049-1052.

EXEMPLARY APPLICATIONS

Compound 27, sometimes referred to herein as "aryl cyclam", as the tetrahydrochloride salt, is a small molecule (mol.wt. = 516.2) with two functionalities. The cyclam moiety has high affinity ($K_s \sim 10^{30}$) and selectivity for Cu(II), the highest of the polyamine (N_4) class of compounds, due to the optimal geometry allowing maximal orbital overlap of coordination bonds. The aryl moiety has moderate affinity, (believed to be $K_s \sim 10^5$), for the serum albumin carrier site for bilirubin, with secondary binding (believed to be $K_s \sim 10^3$) at high concentrations for the hydrophobic amino acid carrier site of serum albumin.

Compound 27 can be used as an orally-administered anti-angiogenesis agent used alone (prophylaxis) to prevent metastatic growth and in combination (adjunct) to potentiate the anti-tumor effects of chemotherapy, radiation therapy and immunotherapy in selected patients with cancer, including, but not limited to, breast adenocarcinoma, prostate adenocarcinoma, colorectal adenocarcinoma, squamous cell carcinoma, bronchogenic carcinoma, ovarian adenocarcinoma, cervical carcinoma, primary and secondary brain cancer, uterine sarcoma, renal cell carcinoma, lung metastases and bladder carcinoma.

Absorption of orally-administered compound 27 is thought to be via the paracellular route within the small intestine, and therefore, driven by concentration and

thermodynamic factors. The high water solubility, small size and moderate binding affinity for serum albumin facilitate absorption. The serum albumin in the blood carries the aryl-cyclam throughout the body.

The targeted albumin site (bilirubin) was selected for several reasons: moderate binding constants can be achieved; a large proportion of unoccupied sites are available; few, if any, other drugs bind there, minimizing the possibility of drug-drug interactions; extraction by the liver is minimized since the albumin-hepatocyte interaction at this site requires a planar, porphyrin structure; and, albumin-bound compound 27 has suitable biodistribution characteristics.

The distribution of compound 27 is thought to be largely limited to the blood pool, except where leaky capillaries at sites of angiogenesis result in extravascular accumulation. Further, high concentrations of albumin are found in the extracellular matrix (ECM) of these angiogenic sites, allowing ligand exchange of compound 27. Within the ECM, the aryl-cyclam remains extracellular, where copper-dependent factors and enzymes are located, and where the uptake by and interaction with cells (and attendant potential toxicity) is minimal.

The metabolism of compound 27 is low, due to the minimum number of potentially reactive groups, the high water solubility, small size and extracellular (and largely intravascular) biodistribution. Further, the half-life within the body (plasma half life believed to be t1/2 ~ 3-6h) prevents accumulation over time.

The excretion pathway is thought to be primarily renal (passive glomerular filtration), due to the high water solubility, small molecular size, and the largely intravascular distribution. A small proportion of the drug will be excreted via the bile (with elimination in the feces), due to liver extraction of free, unbound compound 27 by way of the organic cation carrier protein. The anti-angiogenesis activity is due to the selective, high affinity complexation of Cu(II) by cyclam. Copper is a required co-factor for many angiogenic factors (e.g. KGHK, VEGF, bFGF, etc.), their cell-surface receptors, and the ECM re-modelling enzyme, lysyl oxidase. The stability constants (K_s) for Cu(II) in these biological molecules range from 10^{16} - 10^{21}, while the cyclam moiety has a K_s of 10^{30} for Cu(II). This billion-fold plus difference results in efficient de-coppering and consequent inactivation of these angiogenic molecules. The inhibition does result in down-regulation of the process, but recent studies have shown varying durations among cancer types as well as between individual animals. It seems likely,

then, that continuous drug administration over years will be needed to maintain the dormant state.

The potentiation of chemotherapy with the adjunctive use of anti-angiogenesis agents in animal models of disease is thought to be due to tumor control between cycles and to enhanced permeability of the tumor, which increases exposure to the cytotoxic agents. Response rates, as indicated earlier, are higher for the combination versus either agent alone, as judged by objective tumor response and time to recurrence.

The potentiation of radiation therapy with the adjunctive use of anti-angiogenesis agents in animal models of disease is thought to be due to enhanced permeability of the tumor, which increases the oxygenation. It is well-known that tumors have hypoxic regions which are resistant to radiation-induced death, and that oxygen enhances the generation of cytotoxic free radicals during irradiation.

The potentiation of immunotherapy with the adjunctive use of these anti-angiogenesis compounds may also be facilitated by enhanced permeability. The enhanced permeability within the tumor will facilitate access for both humoral and cellular components of the immune response, and control of tumor growth without marrow toxicity will minimize tumor burden and metastases as the immune response develops over time.

It is also important to not that "treatment-resistance" cannot be developed by the cancer (by clonal selection of somatic cell mutations) since normal cells (e.g., endothelial cells, fibroblasts) and normal factors (e.g., KGHK, lysyl oxidase) are targeted and remain subject to regulation.

6-(4-n-butylphenyl)methyl-1,4,8,11-tetraazaundecane as the tetrahydrochloride salt (an analogue to compound 5 or 13) may be useful as an orally-administered copper chelating ligand for the systemic chelation of excess copper in patents with Wilson's disease, with or without dietary measures to minimize copper absorption from food. It is a small molecule (mol.wt = 452.3) with two functionalities. The tetramine moiety has high affinity ($K_s \sim 10^{24}$) and selectivity for Cu(II), in addition to rapid complexation kinetics under in vivo conditions (37°, neutral pH). The alkylated aryl moiety has low affinity (believed to be $K_s \sim 10^3$) for multiple sites of serum albumin, which yields near-ideal biodistribution characteristics.

Absorption of this orally-administered analogue of compound 5 or 13 is thought to be via the paracellular route within the small intestine, and therefore, driven by

concentration, high water solubility, small molecular size and low affinity, but still significant, binding to serum albumin.

Distribution is expected to have two preferred features, due to the weak, multi-site binding to serum albumin. First, there is a significant "first-pass" extraction by the liver from the portal circulation immediately after absorption from the intestine. Secondly, the remaining fraction within the blood will partition to a significant degree in the extra-vascular space, because of the unbound component (free ligand). The metabolism of this tetramine is expected to be minimal, because of its high water solubility, minimum number of potentially reactive groups (especially unreactive if the ligand is metallated), and the largely extracellular distribution. However, the significant fraction taken up by the hepatocytes may result in some degree of contact with hepato-cellular enzyme systems, with the potential of metabolic interaction prior to excretion. As with any drug, this necessitates the characterization of any metabolites found during pharmacological studies.

Excretion is expected to be approximately equal between the renal and hepato-biliary routes. Discounting the pre-systemic hepatic uptake and elimination (along with bound copper), the plasma $t_{1/2}$ is expected to be about 2h. Total systemic clearance is the sum of hepatic and renal clearance. Importantly, because of the first-pass effect and the high intrinsic hepatic clearance of this compound, even patients with poor hepatic function (e.g. cirrhosis) will be able to excrete excess copper from the liver (as well as other tissues).

The 2,3,2-tetramine chelating moiety has been shown to be more efficient cupriuretic agent than penicillamine and trientine, due to its higher K_s for copper. The cupriuresis and detoxification is expected to be greatly enhanced by the hepato-biliary targeting. The improved efficacy would allow lower dosing of alkyl-tetramine versus conventional drugs, thereby contributing an additional safety margin and improved patient tolerance.

The dosage regimen is expected to be 250 mg, b.i.d. for the initial detoxification, and 100 mg, o.d. for maintenance (life-long) in conjunction with dietary measures or 250 mg, o.d. without dietary control. Experience with conventional chelating agents indicates that the evening dose is most important in control, presumably because most patients consume the largest meal of the day at that time.

6-(4-n-butylphenyl)methyl-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride, an analogue of compound 27, is a small, diffusible molecule which is highly stable to air (0_2) and light (UV) which may be useful as a topically-applied angiogenesis inhibitor for the local control of psoriasis. The amphiphilic nature of the compound facilitates formulation in a water-in-oil emulsion at high concentration. A cosmetically-preferred clear cream may be applied topically to the skin surface where a psoriatic lesion is developing.

Transcutaneous absorption of the compound is expected to occur over several hours. The epidermis of a psoriatic lesion is highly permeable relative to normal skin, due to the impaired barrier function, which relies largely on keratinization. Twice daily application for several days is the expected dosage regimen.

Within the dermis, a high local concentration of the angiogenesis inhibitor is attained. The alkylated aryl moiety facilitates the binding to albumin in the extracellular matrix, with consequent retention of the compound in the immediate vicinity of the lesion. Further, our pilot experiments show significant binding to "immature" collagen undergoing re-modelling, which adds to the retention time. The compound will be absorbed into the systemic circulation with time, where distribution will be similar to that of alkyl-tetramine, with relatively rapid hepatic and renal clearance. The plasma concentration achieved is extremely low and of no appreciable pharmacological effect.

The high intra-lesional concentration of this compound will rapidly inhibit angiogenesis via Cu chelation. Further, *in vitro* cultures of endothelial cells can be inhibited (cytostatic effect) without cytotoxicity with a safety margin of at least 10-fold. The cytostatic effect may prove to be a significant benefit in this therapeutic setting.

Angiogenesis blockade may significantly reduce lesion size and duration, with attendant cosmetic benefit.

EVALUATION OF CELL PROLIFERATION AND CYTOTOXICITY

The process of angiogenesis consists of several discrete, but often coincident, stages through which endothelial cells must progress for the successful completion of a functional vascular bed. One of these stages is proliferation. Therefore, a reasonable isolated assay to perform with the compound was to measure their effect on the cell cycle of endothelial cells in culture.

The effects of various biomimetic targeted chelating agents on [^{3}H]-thymidine incorporation and the *in vitro* cytotoxicity by MTT assay using normal endothelial and tumor cell lines was evaluated in accordance with the following methods.

In a first set of experiments, standard [^{3}H]-thymidine proliferation and MTT cytotoxicity assays were carried out on compounds 13, 15 and 5 using normal cell lines: HMEC (human mammary endothelial cells), HUVEC (human umbilical vein endothelial cells) and HMVEC (human miscrovascular endothelial cells) and tumor cell lines: MDA-MB231 (human breast adenocarcinoma, pleural effusion) and PC-3 (human prostate adenocarcinoma). [^{3}H]-thymidine proliferation assays were conducted in triplicate wells by incubating for 48 hrs. with compounds at various concentrations and pulse-labelling with [^{3}H]-thymidine during the last 18 hrs. of incubation. Radioactivity incorporated into DNA was then counted. The MTT assay was carried out according to method described by Plumb et al., 1982. Control positive compounds: AGM-1470 and Doxorubicin were tested in both assays.

The results are set forth in Table I.

TABLE I

	Endothelial cell						Tumoral cell			
	HMEC		HMVEC		HUVEC		MDA-MB231		PC-3	
	3H	MTT	3H	MTT	3H	MTT	3H	MTT	3H	MTT
Compound 13	>10	>100	>10	>100	>10	72	>10	>100	>10	>100
Compound 15	>10	>100	>10	>95	>10	67	>10	>100	>10	>100
Compound 5	>10	>100	>10	>90	>10	78	>10	>100	>10	>100
AGM-1470	N/D	N/D	0.001	27	0.0007	36	7	54	>10	57
DOXORUBICIN	N/D	N/D	0.004	0.3	0.0003	0.6	0.018	9	0.056	35

HMEC: Human mammary endothelial cell
HMVEC: Human microvascular endothelial cell
HUVEC: Human umbilical vein endothelial cell
MDA-MB231: Human breast adenocarcinoma, pleural effusion
PC-3: Human prostate adenocarcinoma

No significant anti-proliferative effect or cytotoxicity was observed for either endothelial or tumoral cell lines with any of the three compounds tested. Control substances demonstrated the expected results, i.e., marked anti-proliferative effects and cytotoxicity for doxorubicin against all cell lines, and marked anti-proliferative effects

against EC lines and moderate cytotoxicity for all cell lines for AGM-1470 (a.k.a. TNP-470).

None of the three compounds tested showed any significant inhibition of cell proliferation or any cytotoxicity against normal endothelial or tumor cell lines.

None of the three compounds showed significant effect on the cell lines tested. They are relatively much less toxic than AGM-1470 and Doxorubicin.

EFFECT OF BIOMIMETIC TARGETED CHELATING AGENTS ON HUVE CELL COUNTS

The objective of this experiment was to measure total (viable) cell counts following a 4-day *in vitro* exposure to the biomimetic compounds. The following method was used to evaluate the biometic compound on HUVE cells.

Growth arrested quiescent HUVE cells were synchronized and allowed to enter the cell cycle. Cells were exposed to a 5 logs concentrations of each of compounds 3, 13 and 15 consecutively for 4 days by adding freshly prepared compound every day. After 4 days, the viable adherent cells were trypsinized and counted using flow cytometry. Detailed protocol is provided in experimental section.

The following protocol was observed:

Day 0: HUVE cells are plated (24 wells plates) at a density of 10,000 cells per well in the complete media (M199 plus growth factors).

Day 2: Cells are deprived of growth factors by incubation in serum-free Medium 199 containing 0.2% bovine serum albumin for 24 h.

Day 3: Cells are incubated in fresh complete media (3% FBS, 3ng BFGF/ml, 3ng EGF/ml) in the presence of various concentrations of compound.

Day 4 o 6: Fresh dilutions of compounds are added every day.

Day 7: Media is removed and cells trypsinized. Aliquot of 20 µl of cell suspensions are counted with the cytolflurometer Coulter XL.

The results of the effects of the biomimetic compounds on HUVE cells are set forth in Tables II, III and IV.

TABLE II

Compound 13	Conc. (M)	Cell Counts	Average	STDEV	% of control	p=
Control	0	141 114 92 a189	116	25	100.0	
	10^{-6}	a281 115 84 225	105	18	90.5	0.29
	10^{-7}	136 120 87 102	111	21	96.2	0.38
	10^{-8}	152 75 79 95	100	36	86.7	0.22
	10^{-9}	127 57 83 a48	89	35	76.9	0.11
	10^{-10}	122 72 112 157	116	35	100.1	0.26

TABLE III

Compound 15	Conc. (M)	Cell Counts	Average	STDEV	% of control	p=
Control	0	65 86 60 76	72	12	100.0	
	10^{-6}	69 55 55 87	67	15	92.7	0.30
	10^{-7}	67 58 63 48	59	8	82.2	0.12
	10^{-8}	61 75 55 61	63	8	87.8	0.02
	10^{-9}	77 71 66 73	72	5	100.0	0.50
	10^{-10}	102 63 62 55	71	21	98.3	0.47

TABLE IV

Compound 5	Conc. (M)	Cell Counts	Average	STDEV	% of control	p=
Control	0	141 138 129 72	120	32	100.0	
	10^{-6}	132 103 112 126	118	13	98.5	0.47
	10^{-7}	115 90 120 142	117	21	97.3	0.45
	10^{-8}	120 66 102 142	90	25	75.2	0.07
	10^{-9}	90 89 67 113	90	19	74.8	0.15
	10^{-10}	149 109 98 68	106	33	88.3	0.12

The compounds did not show any significant reduction in the total (viable) cell count when used at 10^{-10} to 10^{-6} M concentration range.

No significant contamination with bacterial endotoxin was observed.

No significant cytotoxicity on HUVEC line was observed.

No cell cycle dependent effect on cells was observed.

No effect on cell shape was observed.

None of the compounds tested (compounds 13, 15 and 5) showed any significant effect on cell counts after four days of incubation. The count variation is mainly due to the variation from well to well (some wells contained fewer cells even before drug addition). Microscope observations never revealed any modification in cell shape.

Additional compounds were evaluated for effects using bovine aortic endothelial cells (BAEC) *in vitro*, in accordance to the following methods.

Thymidine incorporation was assayed according to Funk and Sage [1991], *Proc. Natl. Acad. Sci. USA 88*: 2648-2652. Briefly, confluent, contact-inhibited BAEC were arrested by feeding with serum-free medium for 72 h. Cells were then released by brief digestion with trypsin and plated at subconfluent density (approximately 105 cells/well) in 24-well plates in the presence of serially diluted test compounds or phosphatic-

buffered saline (PBS) control in triplicate wells. Compounds were prepared for use by dissolving in PBS at 0.01 M and adjusting the pH with 1 N NaOH.

Twenty hours later the cells were photographed, pulse-labeled with 2 μCi/ml [^{3}H]-thymidine for 2.5 h and were subsequently fixed in ice-cold 5% TCA. TCA-insoluble material was solubilized in 0.4 N NaOH and was assayed in a liquid scintillation counter. To assay for cell death, 25 μl of condition medium was removed from each well prior to fixation to be tested for presence of lactate dehydrogenase (LDH) using the kit from Bio-Analytics (Palm City, FA).

Inhibition of [^{3}H]-thymidine incorporation by BAEC*

Compound	IC_{50}
13	0.2-0.5 mM
15	0.1-0.3 mM
21	0.2-0.5 mM
11	0.4-0.6 mM
25	0.3-0.6 mM
27	0.2-0.4 mM

* Data are based on the mean of triplicate wells from two separate experiments.

LDH Activity In Medium From BAEC Cultures

Only compound 15 showed any increase in LDH activity (outside the cell) over the concentrations tested. The other compounds did not result in any detectable activity, and therefore no evidence of cell death.

EVALUATION OF ANTI-ANGIOGENIC EFFICACY OF THE BIOMIMETIC COMPOUNDS

The anti-angiogenic efficacy of the biomimetic compounds 5, 13 and 15 on growth factor induced neovascularization in a meshed-CAM Assay was evaluated in accordance with the following method.

The compounds anti-angiogenic activity on growth factor induced neovascularization was assessed in the meshed - CAM assay. The compounds were assessed at 160, 80, 40 & 0 μg/disc and the inhibition of angiogenesis quantitated using image analysis software.

The methodology was carried out according to Arispe et al., *Molecular Biology of the Cell 6*, 327-343 (**1995**), and for microvascular density analysis, the images were collected with a Sony CCD camera and quantitated with NIH 1.5 program image analysis software.

The results are set forth in Table V. The numbers presented in Table V indicated the percentage of inhibition of angiogenesis after normalization with the negative control (vitrogen alone) and assuming that VPF (a.k.a VEGF) mesh provides the maximum angiogenic response.

TABLE V

Compound	Time Dosage 40µg	16h 80 µg	160 µg	40 µg	24h 80 µg	160 µg	40µg	36h 80µg	160 µg	40µg	72h 80µg	160 µg
13	1.5 %	2.9 %	3.1 %	9%	27 %	28 %	13%	36%	41%	12%	59%	62%
15	17 %	47 %	50 %	29 %	67 %	79 %	23%	54%	78%	21%	56%	73%
5	2.5 %	12 %	13 %	12 %	13 %	36 %	16%	11%	24%	13%	16%	31%

The results demonstrate that all 3 compounds display anti-angiogenesis activity although with varying potency (15 > 13 > 5). The anti-angiogenesis activity was dose-dependent, and increased with an increase in dose. The amphiphilic compounds (13,15) were more potent than the more hydrophilic compound (5).

EVALUATION OF ANTI-ANGIOGENESIS ACTIVITY IN THE DORSAL AIR SAC ASSAY

The anti-angiogenesis activity of the biomimetic compounds (13, 15 and 5) in the dorsal air sac assay of mice was evaluated in accordance with the following method.

In brief, mice were implanted with a sub-cutaneous air sac with diffusion chambers containing HT-1080 human fibrosarcoma cells (secreting approx. 77 ng/mL VEGF/day). The cells were sourced from the ATCC and used according to Asumo et al., *Cancer Res.* 55 5296 (1995) and Kondo et al. *BBRC* 194 1234, 1993.

The test compounds were administrated by oral gavage b.i.d. and the degree of angiogenesis measured on day 5.

The following protocol was used in the dorsal air sac assay:

a. Mice (CD-1) were initially maintained for 3 days prior to treatment on a low copper synthetic diet containing < 1 ppm of copper.

b. Mice (=4/Gr.) were implanted in the s.c. made air sac with diffusion chambers containing HT-1080 fibrosarcoma cells (secreting approx. 77 ng/ml VEGF).

c. Mice were dosed by oral gavage (twice a day at 6:30 a.m. & 6:30 p.m. before and after availability of low copper food chow given at 8:30 a.m. until 4:00 p.m.). The compounds are given at 160, 80, 40 & 0 μg/mouse for 4 consecutive days (d 1-4). Additional group of mice were injected with 160 μg of each compound s.c. directly into the air sac. Control positive mice were injected with AGM-1470 at 100 mg/kg s.c. in the air sac from d 1-4. Control negative mice were injected with the vehicle alone. All mice were bled and sacrificed on day 5.

The results in the compound 13 test set showed very good neovascularization in the control negative (vehicle-treated) group in the skin area in contact with discs. A nearly complete inhibition of neovascularization was observed in the skin areas of all control positive treated [AGM-1470 at 100 mg/kg s.c. (q D 1-4)] mice. A clearly positive inhibition of neovascularization was observed when compound #13 was given by oral gavage at 80 and 160 μgs doses respectively. No effect was evident following treatment

with 40 μg dose by oral gavage, while s.c. injection of 160 μg directly in the air sac in the vicinity of the disc was relatively less effective compared to the oral route of administration.

The results for the compound 15 test set showed good angiogenesis induction in the control negative (vehicle-treated) group and complete disappearance of neovascularization in the control positive group of mice treated with AGM-1470 at 10 mg/kg s.c. (q D 1-4). A clearly positive effect with significant inhibition of neovascularization was observed following treatment with 160 μg dose by gavage in all 4 animals. Treatment with 80 μg dose showed a lesser effect and 40 μg had no effect in inhibiting angiogenesis. Treatment with 160 μgs s.c. by direct injection into the air sac had no effect.

The results for compound 5 test set showed very good neovascularization in the control negative (vehicle-treated) group and good neovascularization inhibition in the control positive (AGM-1470 treated) group. The only positive angiogenesis inhibition was observed following gavage treatment with 160 μg dose. This was less effective compared to treatment with 80 or 40 μgs respectively. Treatment with 160 μgs s.c. by direct injection into the air sac had an intermediate effect with partial inhibition of angiogenesis.

The results of the dorsal air sac assays are summarized in Table VI.

TABLE IV

*Degree of Angiogenesis (mean of 4 mice)

Groups:	(cpd #13)	(cpd #15)	(cpd #5)
1. Control negative (vehicle alone s.c.)	4,4,5,5,(4.50)	5,5,5,4 (4.75)	5,5,5,4 (4.70
2. Control positive (AGM-1470 100 mg/kg s.c.)	0,1,1,1, (0.75)	0,0,0,1 (0.25)	0,0,101 (0.50)
SynChem Cpds.			
3. Oral gavage 40 µg/mouse	5,5,4,3 (4.25)	4,4,4,4 (4.00)	4,4,5,5 (4.50)
4. Oral gavage 80 µg/mouse	2,2,3,3,(2.50)	3,2,2,2 (2.25)	4,4,3,5 (4.00)
5. Oral gavage 160 µg/mouse	1,2,2,1 (1.50)	1,1,1,2 (1.25)	3,3,4,4 (3.50)
6. S.C. Injection 160 µg/mouse	3,4,4,2 (3.25)	4,3,4 (3.75)	3,4,4,3 (3.50)
7.**Chamber alone	0,0,0,1 (0.3)	N.D.	N.D.

* -The degree of angiogenesis was graded on a scale of 0 negative, 1(<5%), 2 (5-25%) , 3 (25-50%), 4 (50-90%) 5 (>90%) – representing percentage of the area of capillary network covering the area in contact with the disc on day 5.

** -Millipore chambers containing MEM medium alone (without tumor cells).

EVALUATION OF ANTI-ANGIOGENESIS ACTIVITY IN THE QUAIL CAM ASSAY

This assay represents a state-of-the-art protocol, in which the growth and development of blood vessels in the embryonic quail CAM (chorioallantoic membrane) is quantified by a combination of fractal analysis, vessel density, and/or frequency of branch-point genesis. A major advantage of this assay is that the compound can be administered in solution over the entire surface of the CAM (the quail embryo can accommodate a wide range of concentrations of testable compounds). One can immediately discern changes in vessel morphology, in vessel leak, and in frequency of angiogenic sprout formation. The quantification of changes in angiogenesis over a period of 24 to 48 hours is achieved by a new methodology, in which the increase or decrease in vessels is measured by fractal analysis of binarized images as well as by subsequent calculations of vessel density. In these studies the changes in vessel density were limited to vessels of the arterial tree, as the venous circulation tends to lose its blood upon sacrifice of the CAM (and vessels are therefore more difficult to discern).

The assay was conducted according to:

Parsons-Wingerter, P., A.S. Greene, A. Milaninia, A. Redlitz, J.I. Clark and E.H. Sage (1997) Angiogenesis in the Avian Chorioallantoic Membrane I. A Novel Morphometric Assay of Stimulation by bFGF and Inhibition by Angiostain. Submitted for publication.

Fertilized Japanese quail eggs (*Coturnix coturnix japonica*), cleaned with 70% ethanol, were maintained at 37°C until embryonic day 3. The shells were then opened with a razor blade and sterile scissors, the contents transferred into 6-well tissue culture plates, and returned to 37°C. At embryonic day 7, 0.5 ml PBS containing test compounds in a range of concentrations from 0.1 - 4 mM, was applied drop-wise to cover the surface of viable embryos which were then incubated another 24h. The quail are not an inbred strain and there was some variability between embryos in the rate of growth and response to the test compounds. CAMs from eyeless or under-sized embryos were not used.

On day 8, embryos were fixed with 5 ml of pre-warmed 2% gluteraldehyde, 4% paraformaldehyde in PBS for 48 h at room temperature. Alternatively, embryos were given a second dose of test compounds in PBS and then fixed 24 h later (embryonic day 9).

Fixed CAMs were dissected from the surface of the embryo and mounted on glass slides using 10% polyvinyl alcohol, 25% glycerol in 0.5 M Tris, pH 8.5. The dried, mounted CAMs were then photographed with a Nikon Microphot-SA photo microscope at 10 x magnification on 35 mm slide film and the images scanned with a Nikon LS-1000 film scanner into an Adobe Photoshop file for processing. No staining was necessary, as the arteries retained enough blood to render them semi-opaque. To quantify arterial branching, slides were projected in a Reflecta Diamator slide previewer with an 8 x 11 inch grid superimposed on the screen and a field corresponding to a 0.5 cm^2 area of the CAM was scored for primary, secondary, and tertiary branch points. Three membranes were scored per test solution. A total of nine experiments were performed, four using 21, and six with 22 (one experiment included compound 21 pre-loaded with Cu, as a control), and five experiments using 27.

Results:

A decrease in arterial vessel density to some degree was seen in 80% of CAMs treated with compounds 27 and 21 at concentrations of 0.2 mM and above, based on general morphological examination (FIGs. 1-4). Branch points counting of one experiment showed a 30% decrease in total branch points in 8 day CAMs (Fig. 5A). Branch point

analysis of one experiment revealed a proportionate increase in primary and reduction in tertiary branching (Fig. 5B).

Although it cannot be stated with certainty the actual concentration of compound 27 that becomes available to the CAM, a nominal estimate would be approximately 30-50% of the indicated test dose.

Upon microscopic examination, 9 day CAMs treated for 48 h (two applications) appeared to have shorter primary and secondary vessels, but no significant reduction in total branch points. Analysis of degree of branching, however, revealed a significant reduction in tertiary branching in 9 day CAMs which was dose-dependent (Figs. 6a and 6b).

CAMs treated with compound 27 at 0.5 mM, or greater, tend to have shorter, thicker primary branches, compared to controls, and, in some membranes, a larger number of tiny vessels branching off the main vascular tree. Compound 21 pre-loaded with Cu (23) had little or not apparent effect on vessel density (data not shown).

The 24 h assay using 7-8 day CAMs showed less variability and more dramatic changes than the 9 day CAMs fixed after 48 h (Fig. 7). The 48 h assay was problematic because of the decreased viability of all embryos at longer incubation times. There was no significant decrease in viability of the embryos, compared to PBS controls, at doses of compounds up to 2 mM. Quail embryos are known to be exquisitely sensitive to toxic materials, for example, endotoxin at pM concentrations.

EVALUATION OF COMPOUNDS IN TUMOR MODELS

Mouse Melanoma Tumors

Objective:

To evaluate the effect of compounds 21, 22, and 27 on the growth of primary tumors in mice.

Method:

These experiments employed published methods (Chambers et al., Cancer Res 42, 4018-4025, 1982). All mice were given subcutaneous injections of mouse melanoma cells to initiate primary tumor growth on the flank of the animal. Mice were divided into seven groups of ten animals each. Starting one (1) day following injection of the tumor cells, each treatment group received twice daily oral administration (gavage) of one of

compounds 21, 22, or 27 at either high (300 μg/day) or low (150 μg/day) doses, or water (control mice). A summary charge of treatment group follows:

Group	Tumor Cells	Gavage	Dose (μg/day)
T	yes	Compound 21	Water only
4-L	yes	Compound 21	150 (Low)
4-H	yes	Compound 22	300 (High)
6-L	yes	Compound 22	150 (Low)
6-H	yes	Compound 27	300 (High)
7-L	yes	Compound 27	150 (Low)
7-H	yes	Compound 27	300 (High)

The size of the growing tumor of each animal was measured every two days. The experiment was terminated 21 days after the start of treatment with the compounds. All surviving mice were killed and their tumors excised, weighed, measured and fixed in formalin for preservation. Final tumor volumes were calculated and the growth rate of each tumor during the course of the study were graphed. The results of each treatment group were compared with the control group.

Results:

Comparison of treated and control mice at 21 days showed significantly decreased tumor weight in mice treated with compound 21 (150 μg/day) compared to control animals ($P<0.05$) (Fig. 8). There was a trend to decreased tumor weight in animals treated with compound 27, however, the difference was not statistically significant ($P\leq 0.1$). These decreases in tumor weight were reflected in the tumor volume: mice treated with both compound 21 (150 μg/day) and 27 (300 μg/day) had smaller tumors than did control animals ($P\leq 0.1$) (Fig. 9). In contrast, administration of compounds 21 at 300 μg/day, 22 at either 300 or 150 μg/day, and 27 at 150 μg/day did not result in decreases in tumor weight and volume.

The rate of tumor growth during the experiment was analyzed and graphed (FIGs. 10-12). Tumors in mice treated with low doses of compound 21 (150 μg/day), and high doses of compound 27 (300 μg/day), were smaller than control at early stages of tumor

growth; these results are consistent with the results for final tumor volume and weight. In addition, there were smaller but suggestive decreases in tumor volume compared to control, in early stages of tumor growth in mice treated with compound 22 (150 μg/day).

Conclusions and Remarks:

Test compounds 21 and 27 inhibited the growth of primary tumors in mice.

Mice treated for three weeks with the compounds 21, 22, and 27 showed no visible signs of toxicity.

HUMAN PROSTATE TUMORS

Objective:

To evaluate the effect of compound 27 on the growth of primary human prostate cancer tumors in mice.

Method:

These experiments employed published methods (Behrend, Craig, Denhardt, and Chambers, Cancer Res. 54, 832-837, 1994; O'Reilly et al., Nature Medicine 2, 689-692, 1996). All mice were given subcutaneous injections of human PC-3 prostate carcinoma cells to initiate primary tumor growth on the flank of the animal. Mice were divided into three groups of eight to nine animals each. Starting one (1) day following injection of the tumor cells, each treatment group received twice daily oral administration (gavage) of compound 27 at either low (250 μg/day) or high (500 μg/day) doses, or water (control mice). All mice were maintained on a formulated diet. A summary chart of treatment groups follows:

Group	Tumor Cells	Gavage	Dose (μg/day)
W	1×10^6	Water only	0
7L	1×10^6	Compound 27	250
7H	1×10^6	Compound 27	500

The size of the growing tumor of each animal was measured every three to four days. The experiment was terminated 73 days after the start of treatment with compound 27. All surviving mice were killed and their tumors excised, weighted, measured and fixed in formalin for preservation. Final volumes of subcutaneous tumors were calculated and

the growth rate of each tumor during the course of the study was graphed. The results of each treatment group were compared with the control group.

Results:

A primary tumor formed at the injection site in all control mice. One mouse from the low dose 27 group (250 μg/day) did not form a tumor, and two mice from the higher (500 μg/day) dose group formed no tumors.

Final volumes of tumors in each treatment group were divided into two size categories to assess any effect of compound 27 on tumor size. Eighty percent of control tumors were larger than 300 mm^3 (Fig. 13A). In contrast, 78% of tumors from mice treated with compound 27 (500 μg/day) were smaller than 300 mm^3 (Fig. 13C). Treatment of mice with the low dose of compound 27 resulted in an almost equal distribution of tumors in both size categories (Fig. 13B). The same results were obtained for final tumor weight: whereas 80% of control tumors were larger than 300 mg (Fig. 14A), 78% of tumors from compound 27 mice (500 μg/day) were smaller than 300 mg (Fig. 14C).

Growth curves were graphed for each treatment group. The curves show that treatment of mice with compound 27 at 500 μg/day resulted in decreased tumor growth as measured by tumor volume (Fig. 15). There was a statistically significant decrease in tumor volume in compound 27 (500 μg/day) mice compared with control mice at day 52 ($P<0.05$). A comparison of tumor volume at the end of the study showed that, while not statistically significant, there was a trend to smaller tumor volume in mice treated with 500 μg/day compound 27 ($P<0.08$). In contrast, compound 27 at 250 μg/day had no significant effect on tumor volume at any time during the study (Fig. 16).

Compound 27 at 500 μg/day resulted in decreased final tumor volume and weight of human PC-3 prostate cancer cells growing in mice. Compound 27 at 500 μg/day resulted in a trend to reduced tumor growth rate. Compound 27 did not result in any toxicity to the mice, at either dose tested.

MEASUREMENT OF SERUM Cu IN TEST ANIMALS

Objectives:

To measure blood copper and zinc levels in mice (SCID) with implanted human prostate (PC-3) tumors, and treated with compound 27.

To determine if final tumor volume is related to blood copper or zinc levels.

Experimental Methods:

Blood samples were collected from mice at the termination of the above discussed human prostate tumor study, from the three groups of mice: treated with compound 27 at high (group 7H) or low (group 7L) dose, on water only control (group W). Blood samples were stored frozen until analysis. Copper and zinc levels were determined using ICP-MS with Ga as an internal standard.

Results:

Measured copper and zinc blood levels for each mouse are shown in Table VII, along with final tumor volume.

There was a statistically significant positive correlation between blood copper level and final tumor volume (regression analysis, $r^2=0.521$, $P<0.001$) (Fig. 17).

The mean blood copper levels for the 7H and 7L groups did not differ statistically from the control values (t-test, $P=0.138$, 0.378, respectively), due to the variability in the control values. However, more of the low blood copper values and small tumor sizes were found in the 7H group, and more high blood copper values and large tumor sizes in the control group, with the 7L group intermediate (see Fig. 1).

There was not significant correlation between blood zinc level and final tumor volume (regression analysis, $r^2=0.00638$, $P=0.731$) (Fig. 2).

Conclusions and Remarks:

Blood copper levels were positively related to final tumor size, confirming the relationship between copper levels and angiogenesis. Low blood copper levels were associated with small tumor volume, and high blood copper levels were associated with large tumor volume.

Variability in control mouse copper levels likely masks effects due to treatment with compound 27.

Blood zinc levels were not related to final tumor size.

TABLE VII

Mouse #	Blood Copper Levels (ppb; ng/ml)	Blood Zinc Levels (ppb/; ng/ml)	Final Tumor Volume (mm^3)
7H2-1	416.23	1270.04	85
7H2-2	474.50	4574.27	484
7H2-3	556.27	2622.89	187
7H2-4	614.32	2170.37	527
7H1-1	484.84	1818.45	0
7H1-2	437.76	1409.12	249
7H1-3	364.47	1310.00	0
7H1-4	469.66	1360.36	263
7H1-5	462.86	1475.61	187
7L2-1	495.56	1536.45	400
7L2-3	572.67	1821.21	263
7L2-4	514.25	1233.91	590
7L1-1	507.37	1772.42	65
7L1-2	516.76	1542.69	548
7L1-3	434.43	1617.92	0
7L1-4	601.47	1500.22	692
W2-1	929.50	1383.12	881
W2-2	473.23	1385.72	463
W2-3	495.65	1516.84	484
W2-4	665.84	1638.12	463
W1-4	412.15	1304.29	58

ADMINISTRATION OF THE BIOMIMETIC TARGETED CHELATING AGENTS

The chelating ligands of the invention are administered to patients by oral, parenteral, or topical routes in a pharmaceutical vehicle. Dosage depends on the composition of the selected chelating agent, on individual patient copper levels or on the surface area of skin to be treated. In general, dosage will be in the range of about 0.0001-0.05 mmol/kg of body weight per day with oral administration requiring the highest doses and topical administration requiring the least. Depending upon the excretion rate of the selected chelating agent, administration will vary from 1-3 times daily. The biomimetic compound is preferably administered as an acid salt, the free base or a complex with a displacable metal ion such as zinc.

The compounds of the preferred embodiment are useful in treating Wilson's disease, angiogenesis dependent cancer tumors, psoriasis, and other diseases where angiogenesis causes or exacerabates the disease. However, such compounds may be more suited to treating some of the diseases than for others.

It will be apparent to those skilled in the art that various modifications to the preferred embodiment of the invention as described herein can be made without departing from the spirit or scope of the invention as defined by the appended claims.

The invention claims is:

1. A polydentate amine ligand for chelating copper in mammals corresponding to the formula: $N_x(R)_B$ $aHcY$ wherein Nx refers to a linear, macrocyclic, or caged (strapped macrocyclic) polydentate amine ligand containing up to 60 nonhydrogen atoms, in which x is the number of nitrogen donor groups N, and is a number from 3 to 12, and the nitrogen-donor groups are separated by 2-3 nonhydrogen atoms so that metal binding results in linked 5- and 6-membered chelate rings; HcY is an acid that may be used to partially or fully neutralize the amine groups; C is a number from 1 to 3; a is the number, from 0 to 12, of moles of acid used to convert the polydentate amine to a salt; R is a lipophilic moiety such as an linear, branched, or cyclic alkyl group, aromatic group, heterocyclic group, or a combination thereof, each of which may be optionally substituted with one or more groups such as halogen atoms, ethers, carboxylic acids, amines etc., some of which are donor ligands themselves and can enhance the binding of metal ions such as Cu(II); B is the number of lipophilic moieties R present and is a number from 0 to 12; and R may be attached to a nitrogen donor atom or a nonhydrogen atom.

2. A polydentate amine ligand according to claim 1 corresponding to 6-(3-bromo-6-hydroxyphenyl)methyl-1,4,8,11-tetraazaundecane tetrahydrochloride.

3. A polydentate amine ligand according to claim 1 corresponding to 6-(3-bromophenyl)methyl-1,4,8,11-tetraazaundecane tetrahydrochloride.

4. A polydentate amine ligand according to claim 1 corresponding to 1-(3-bromophenyl)methyl-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride.

5. A polydentate amine ligand according to claim 1 corresponding to 12-(3-bromophenyl)methyl-1,4,7,10-tetraazacyclotridecane tetrahydrochloride.

6. A polydentate amine ligand according to claim 1 corresponding to 12-(4-isopropylphenyl)methyl-1,4,7,10-tetraazacyclotridecane tetrahydrochloride.

7. A polydentate amine ligand according to claim 1 corresponding to 6-(3-bromobenzyl)-1,4,8,11-tetraazacyclotetradecane tetrahydrochloride.

8. A polydentate amine ligand according to claim 1 corresponding to 6-(4-n-butylphenyl)methyl-1,4,7,10-tetraazaundecane tetrahydrochloride.

9. A polydentate amine ligand according to claim 1 corresponding to 6-(4-n-butylphenyl)methyl-1,4,7,10-tetraazacyclotetradecane tetrahydrochloride.

10. A polydentate amine ligand according to claim 1 that is complexed with a positively charged metal ion such as Zn(II), Mg(II) etc. that may be displaced by the more strongly bound Cu(II) ion.

11. A method of chelating copper *in vivo* in animals comprising administering to a mammal a biomimetic compound, characterized in that:

the biomimetic compound is taken up preferentially by hepatocytes as compared with other tissues, or by serum albumin as compared with other plasma proteins;

the biomimetic compound selectively chelates copper, compared to other metal ions;

the biomimetic compound has a stability constant of at least 10^{15} for copper, a solubility of at least 0.001 mmol per liter in normal saline, a molecular weight greater than 250, and a charge of an absolute value of 3 or less at neutral pH; and

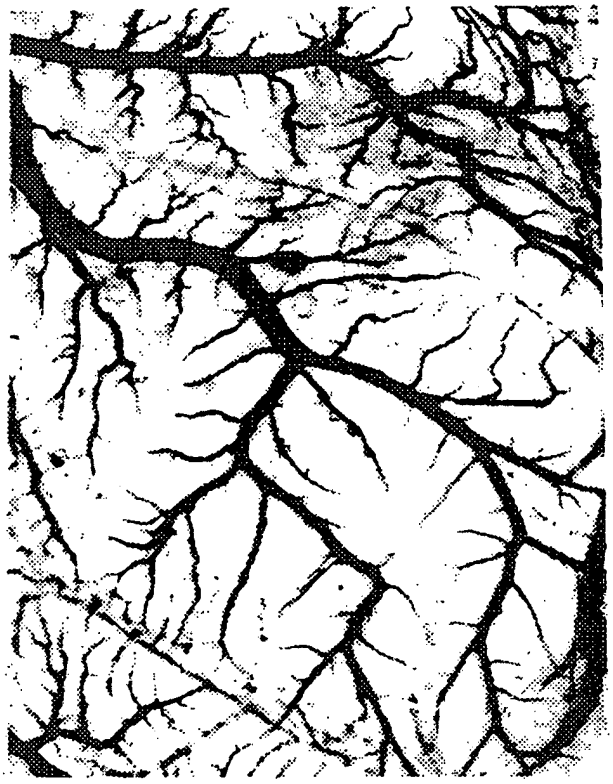
the biomimetic compound contains at least one lipophilic moiety.

12. The method of claim 11, wherein the biomimetic compound is further characterized in that it has a lipophilicity which is sufficiently high to cause it to be taken up in greater amount by normally functioning hepatocytes as compared to other tissues, and by serum albumin as compared to other plasma proteins.

13. A method of chelating copper *in vivo* in animals comprising administering to an animal a biomimetic compound represented by the formula: $N_x(R)_B$ aHcY wherein Nx refers to a linear, macrocyclic, or caged (strapped macrocyclic) polydentate amine ligand containing up to 60 nonhydrogen atoms, in which x is the number of nitrogen donor groups

N, and is a number from 3 to 12, and the nitrogen-donor groups are separated by 2-3 nonhydrogen atoms so that metal binding results in linked 5- and 6-membered chelate rings; HcY is an acid that may be used to partially or fully neutralize the amine groups; C is a number from 1 to 3; a is the number, from 0 to 12, of moles of acid used to convert the polydentate amine to a salt; R is a lipophilic moiety such as an linear, branched, or cyclic alkyl group, aromatic group, heterocyclic group, or a combination thereof, each of which may be optionally substituted with one or more groups such as halogen atoms, ethers, carboxylic acids, amines etc., some of which are donor ligands themselves and can enhance the binding of metal ions such as Cu(II); B is the number of lipophilic moieties R present and is a number from 0 to 12; and R may be attached to a nitrogen donor atom or a nonhydrogen atom.

14. The method of claim 13, wherein said biomimetic compound is taken up preferentially by human hepatocytes, as compared to other tissues, and is preferably taken up by serum albumin as compared with other plasma proteins; and selectively chelates copper as compared to other metal ions.



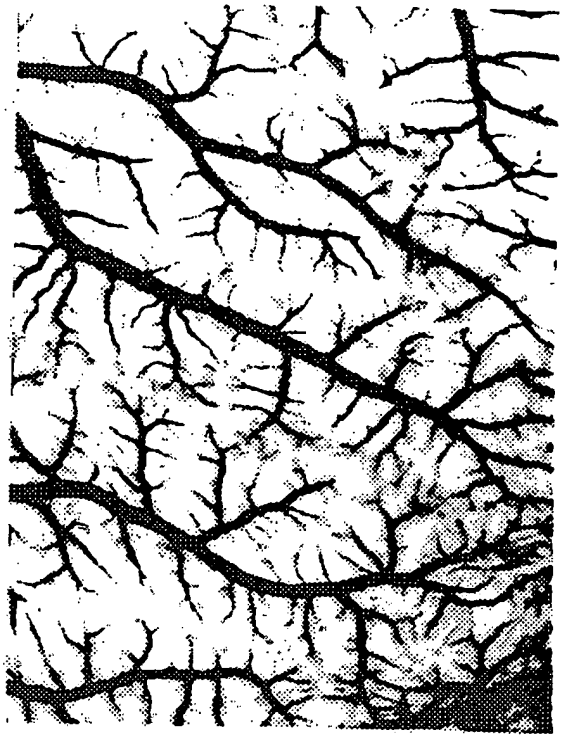
PBS, 48 h, 7-9 d

FIG. 1A

Compound 27, 0.2 mM, 48 h

FIG. 1B

Compound 27, 0.5 mM, 48 h



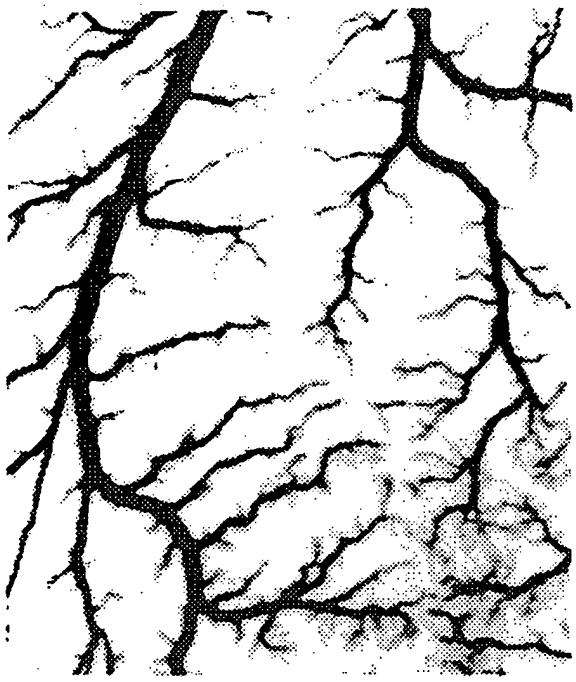
PBS, 24 h, 7-8 d

FIG. 2A

Compound 27, 0.5 mM

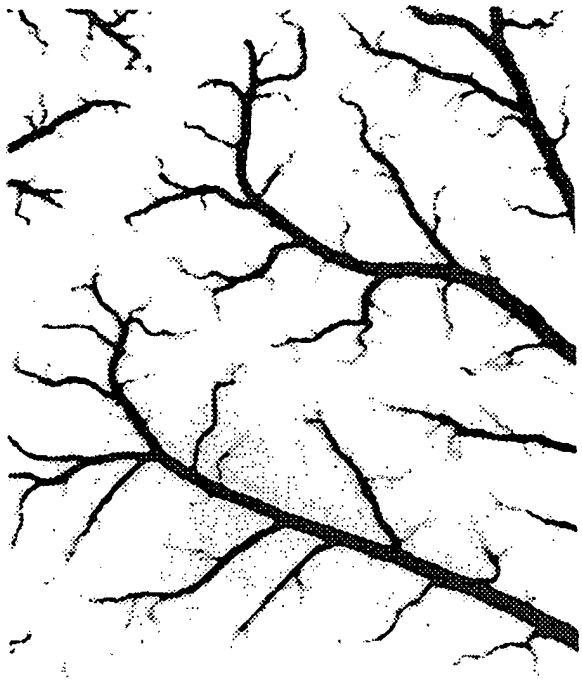
FIG. 2B

Compound 27, 1 mM



PBS, 24 h, 8-9 d

FIG. 3A



Compound 21, 0.2 mM

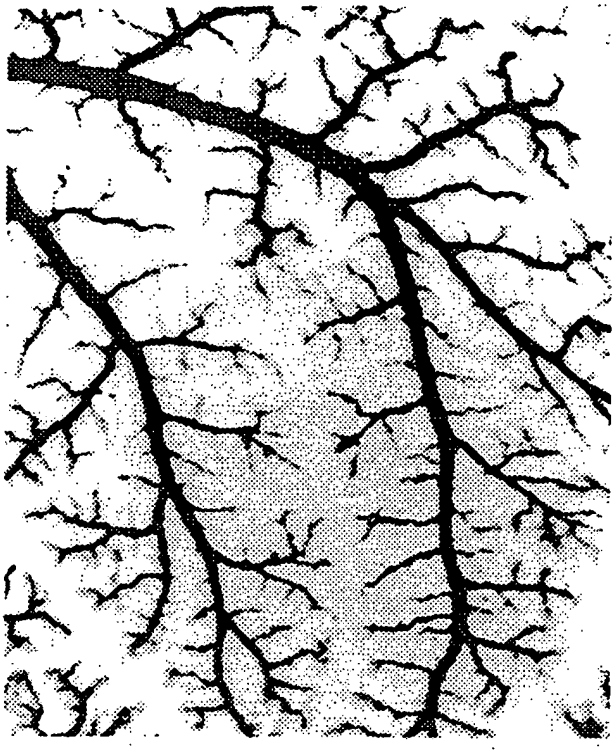
FIG. 3B

Compound 21, 0.3 mM

FIG. 3C

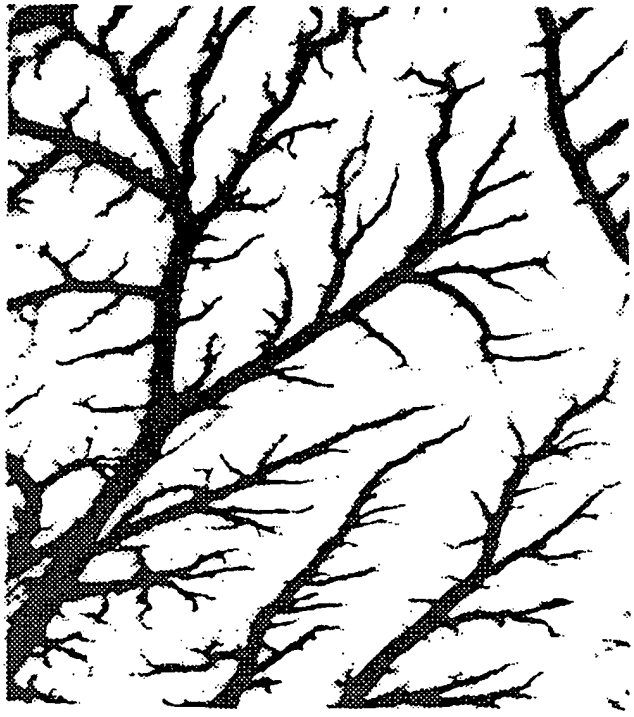
PBS, 24 h, 8-9 d

FIG. 4A



Compound 21, 1 mM

FIG. 4B



Compound 21, 2 mM

FIG. 5A

24 h Treatment with Compound 27
Total arterial branch points per field

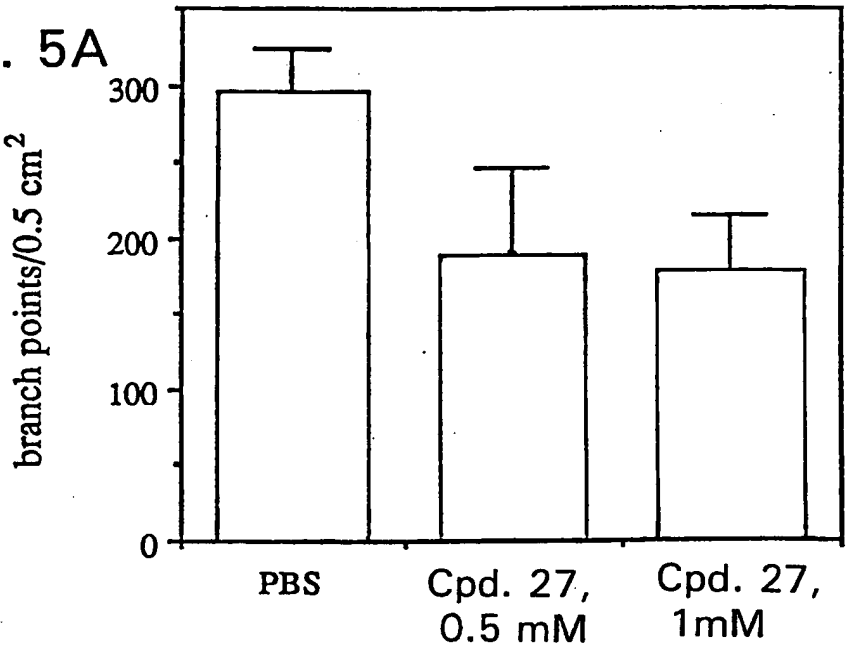


FIG. 5B

Quantification of Arterial Branching in 8d Quail CAMs:
24 h treatment with Compound 27

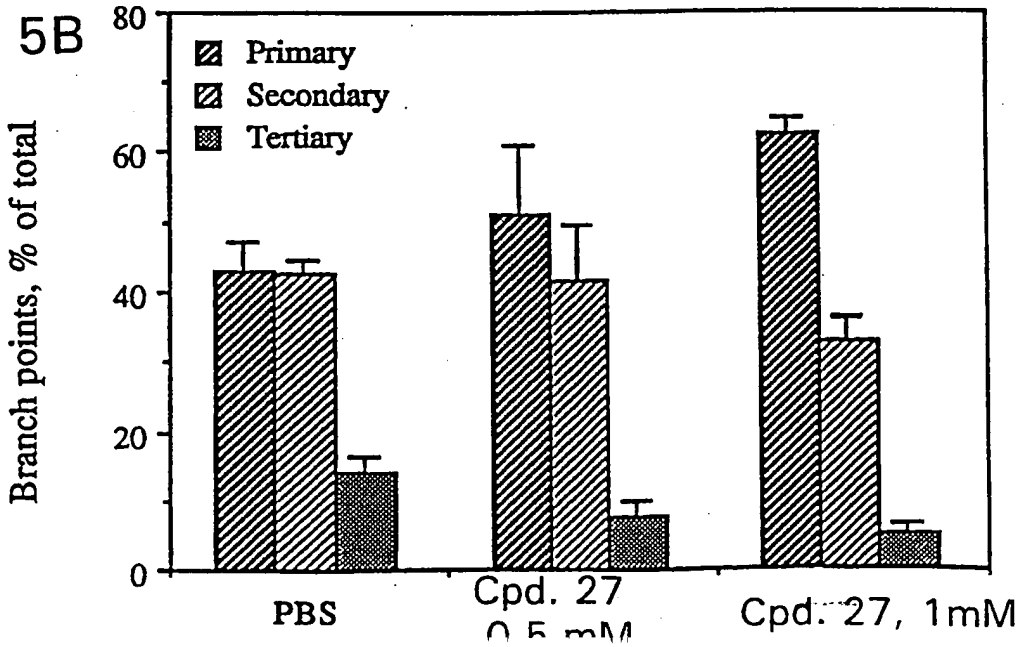


FIG. 6A

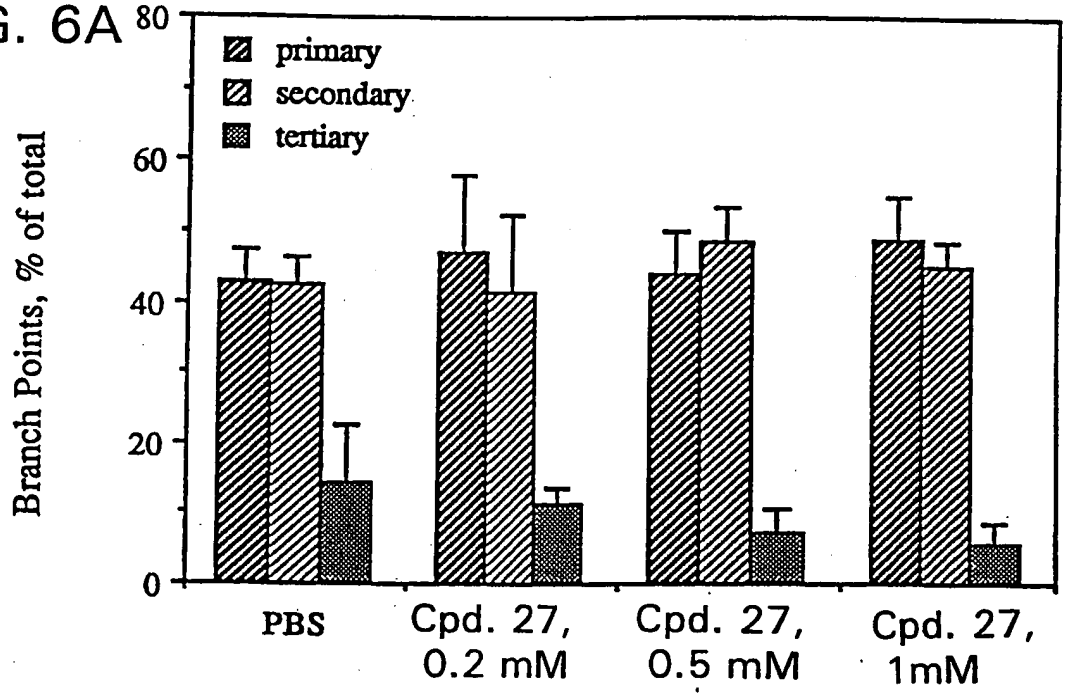

Analysis of Arterial Branching in 9d Quail CAMs
Treated with Compound 27, 2 Applications, 48h
Branch Points, % of total
primary
secondary
tertiary
0
20
40
60
80
PBS
Cpd. 27, 0.2 mM
Cpd. 27, 0.5 mM
Cpd. 27, 1mM

FIG. 6B

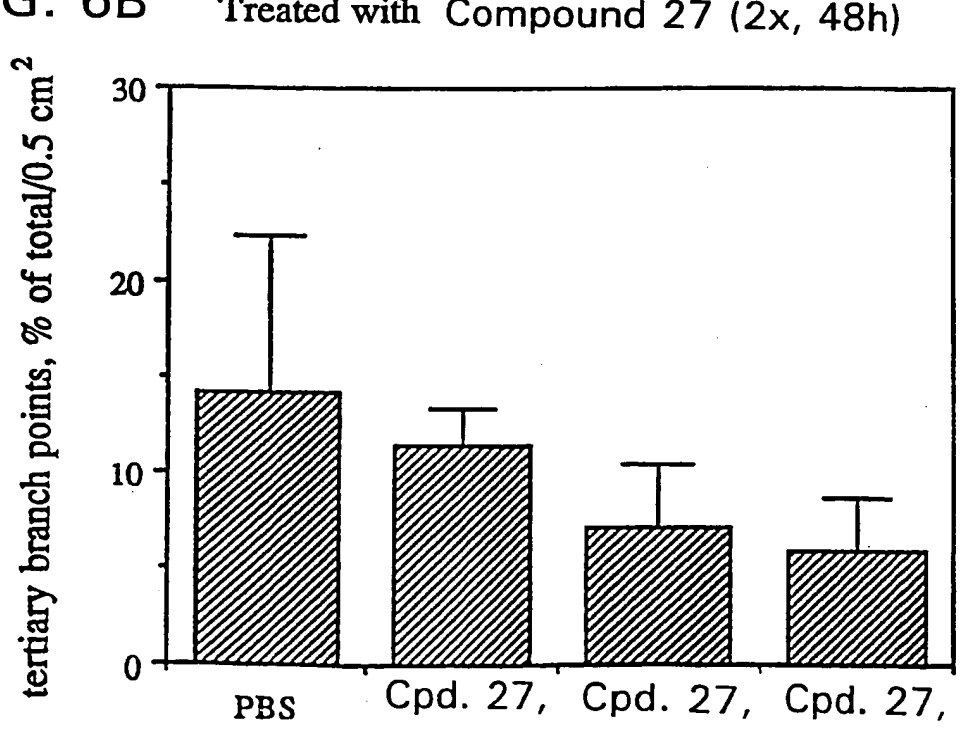

Tertiary Branching in 9d Quail CAMs
Treated with Compound 27 (2x, 48h)
tertiary branch points, % of total/0.5 cm2
0
10
20
30
PBS
Cpd. 27,
Cpd. 27,
Cpd. 27,

FIG. 7

Tertiary Branching in Quail CAMs Treated with Compound 27

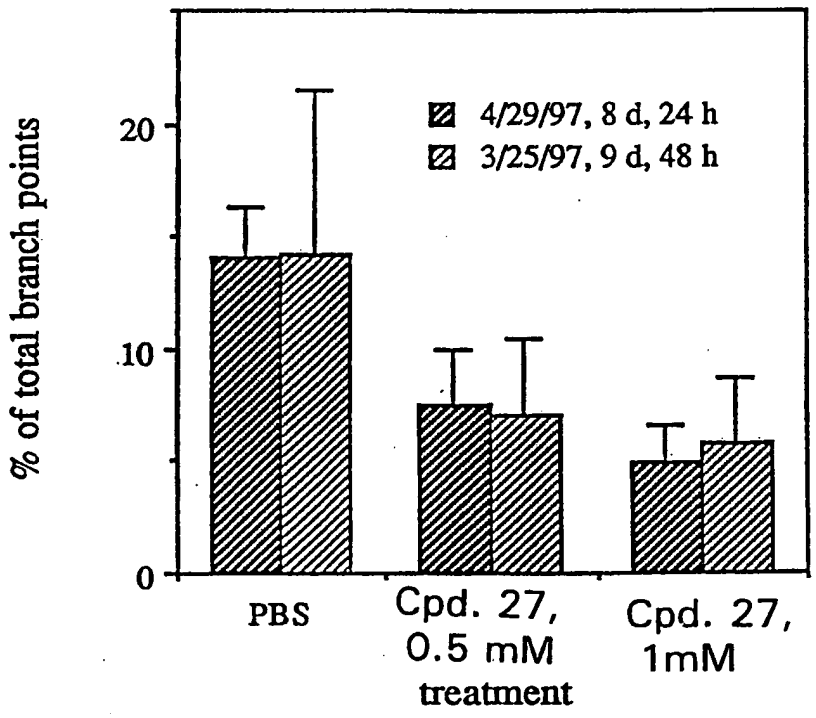


FIG. 8

Effect of Compounds 21, 22 and 27 on Tumor Weight

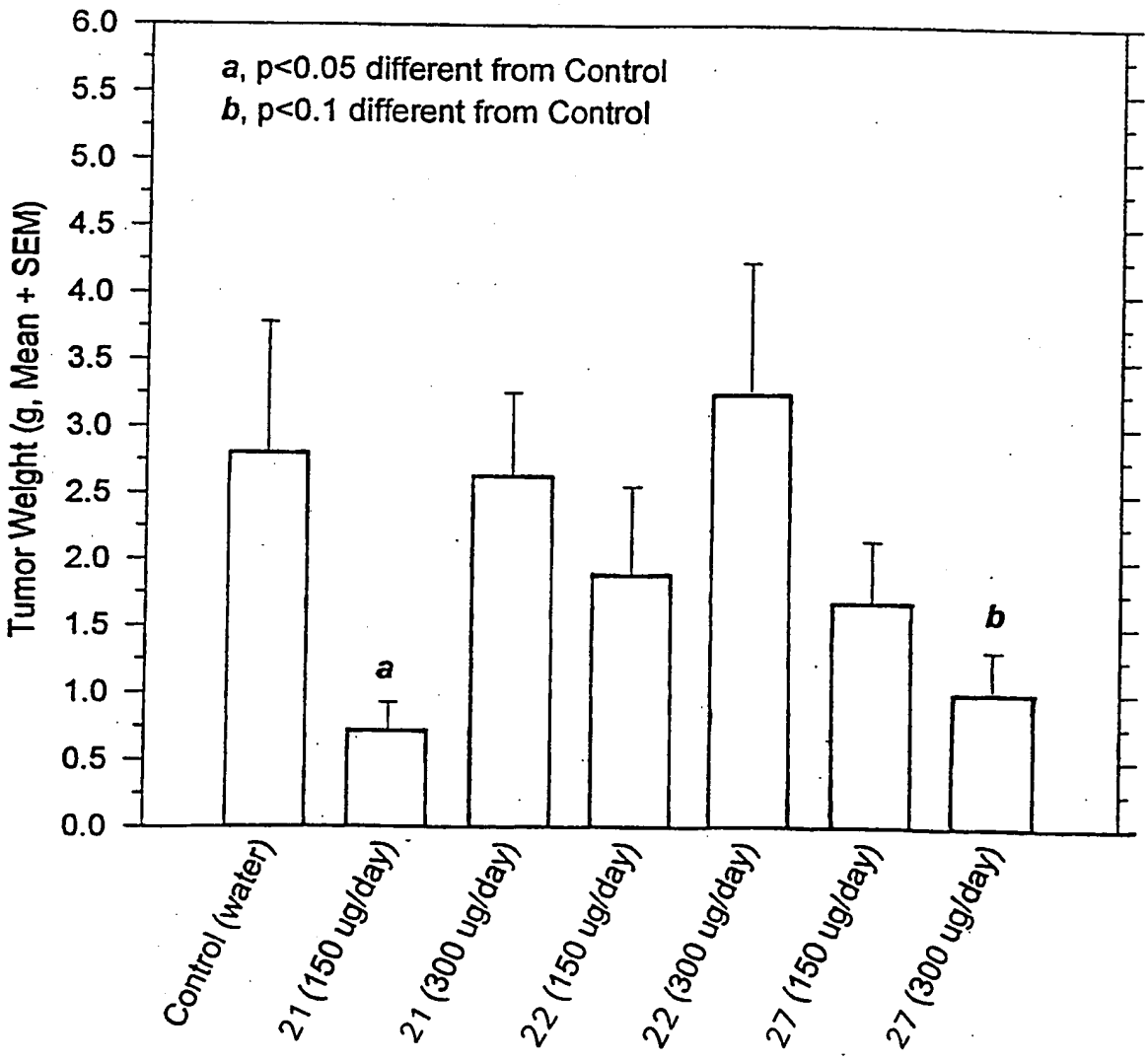


FIG. 9

Effect of Compounds 21, 22 and 27 on Tumor Volume

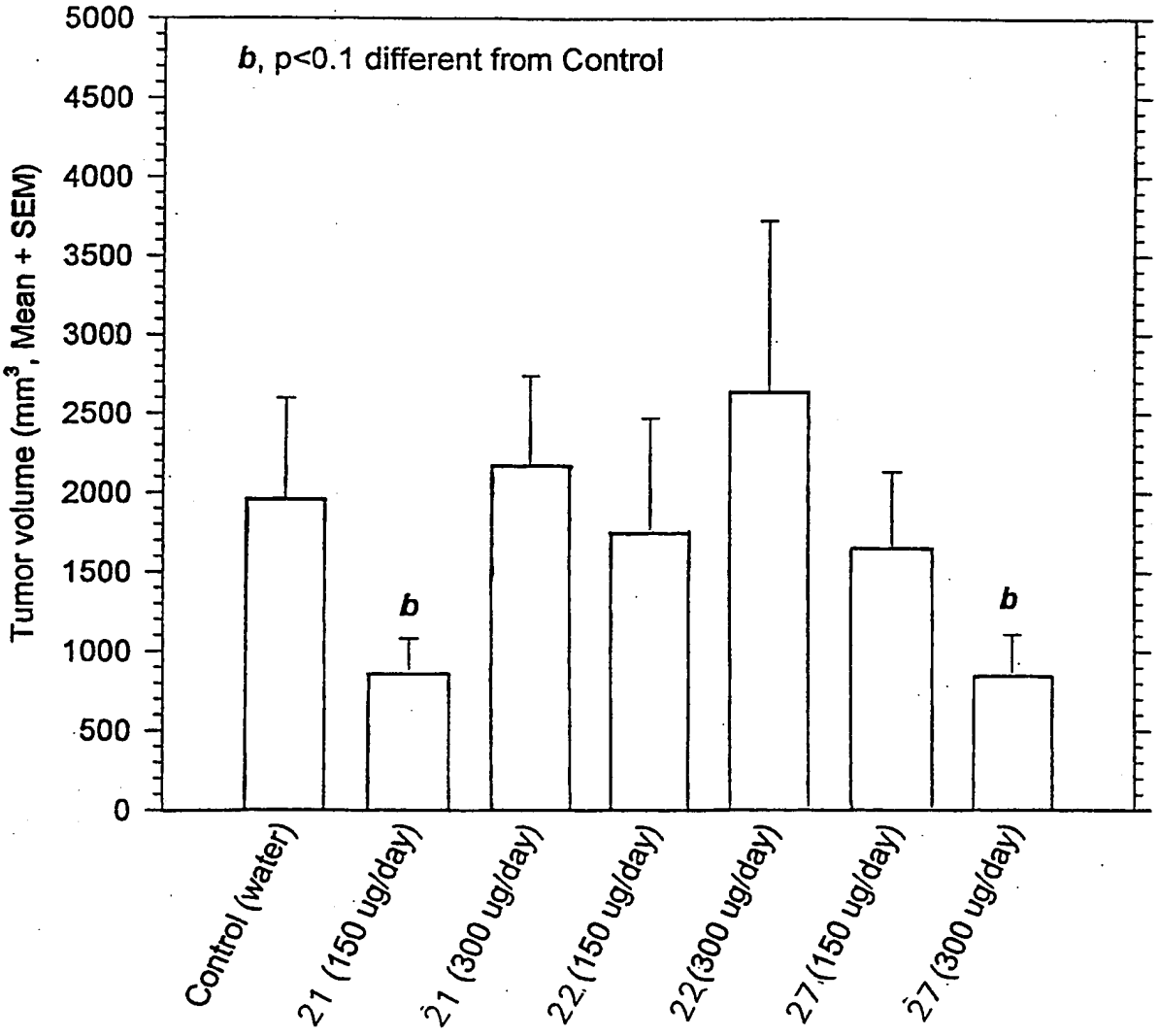


FIG. 10 Effect of Compound 21 on Tumor Volume over Time

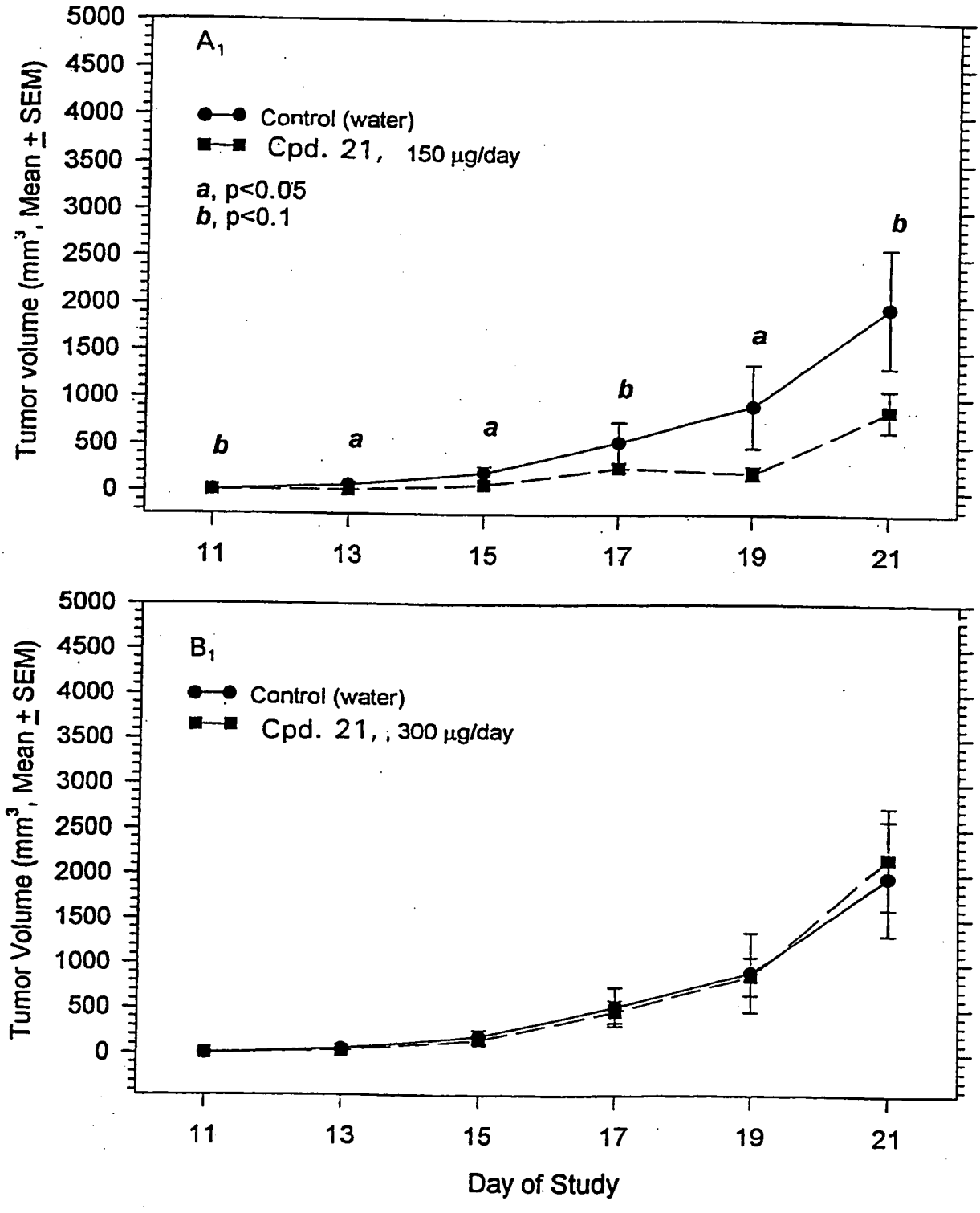


FIG. 11 Effect of Compound 22 on Tumor Volume over Time

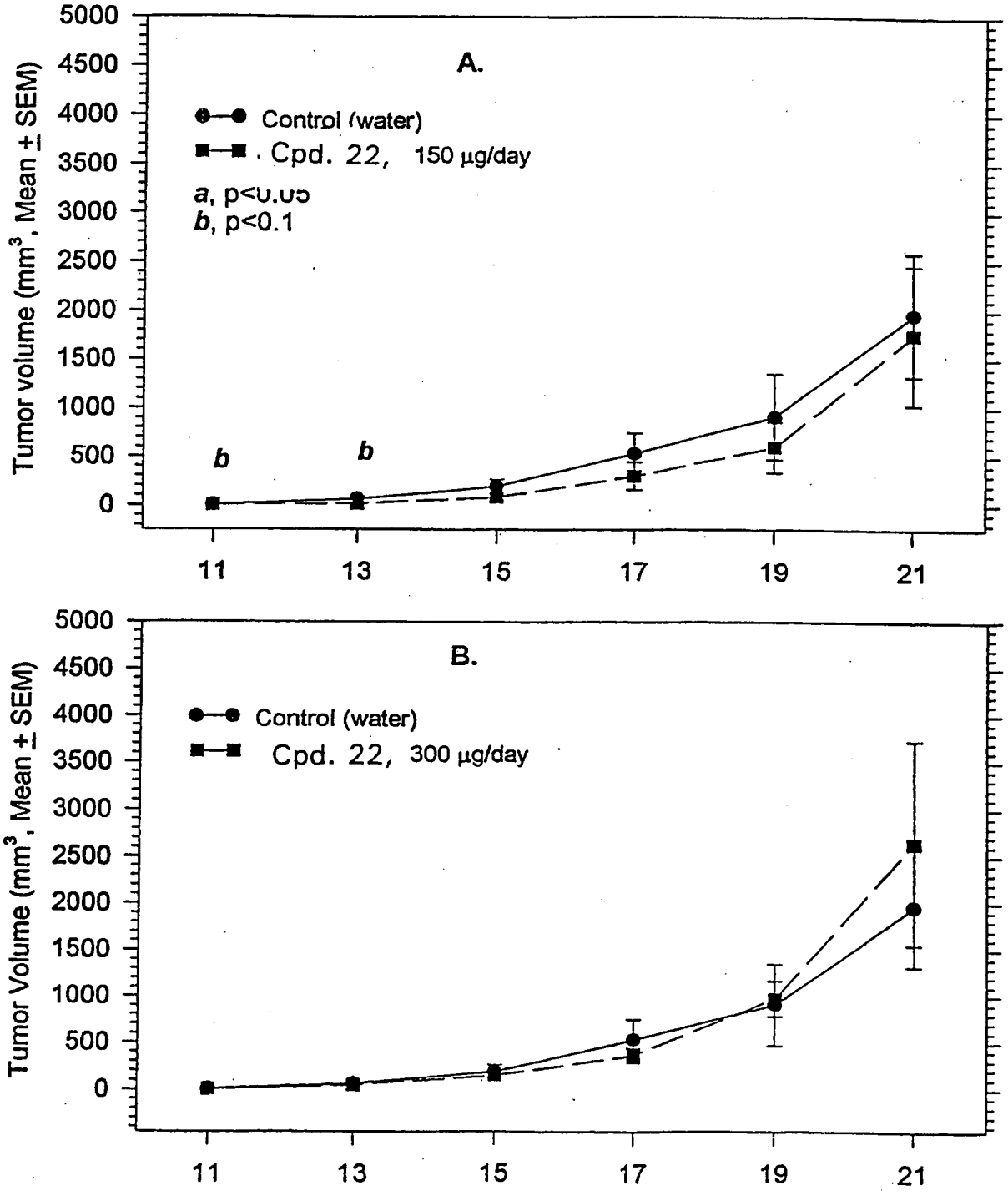


FIG. 12 Effect of Compound 27 on Tumor Volume over Time

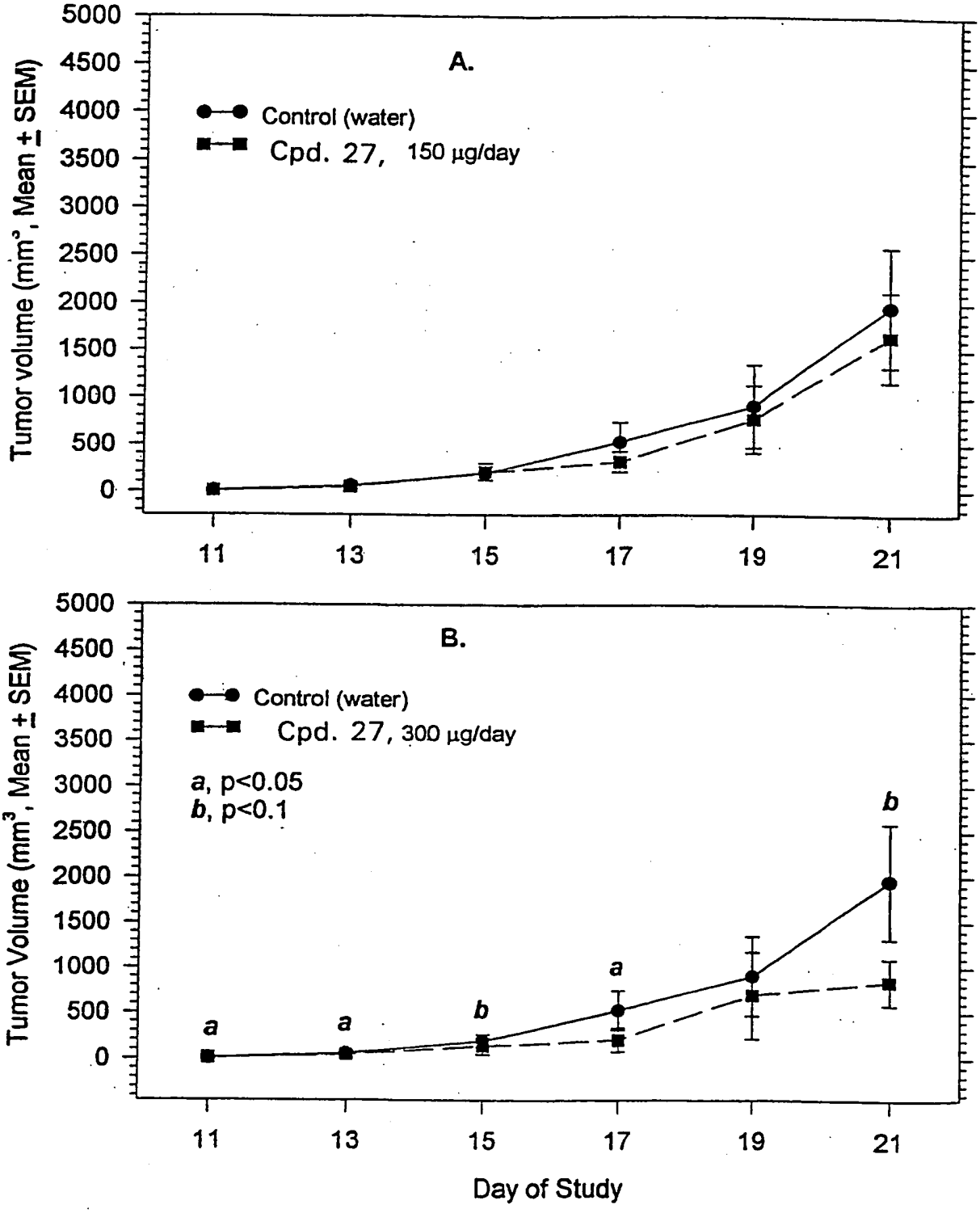


FIG. 13 % Frequency Distribution of Tumor Volume

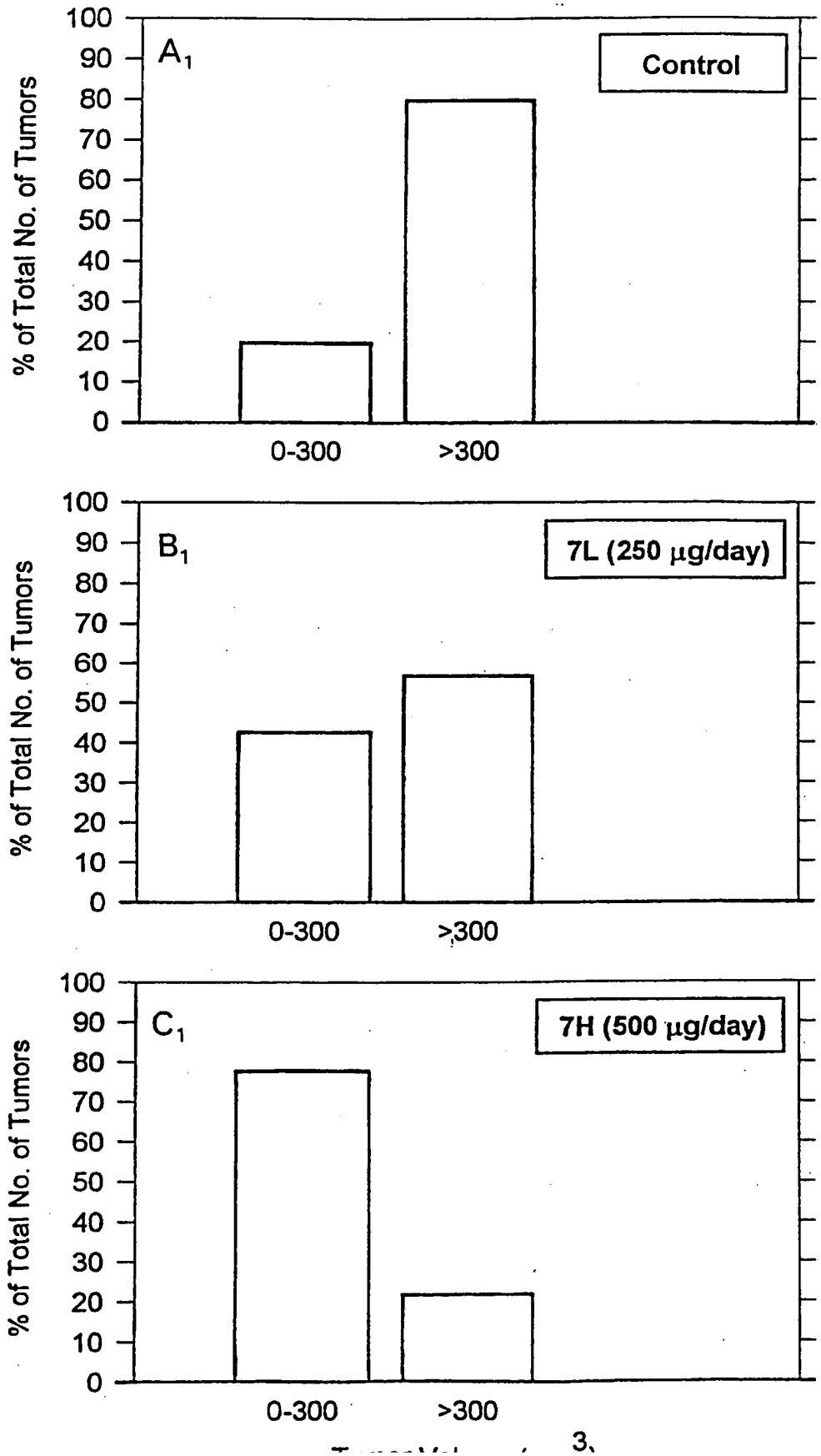


FIG. 14 % Frequency Distribution of Tumor Weight

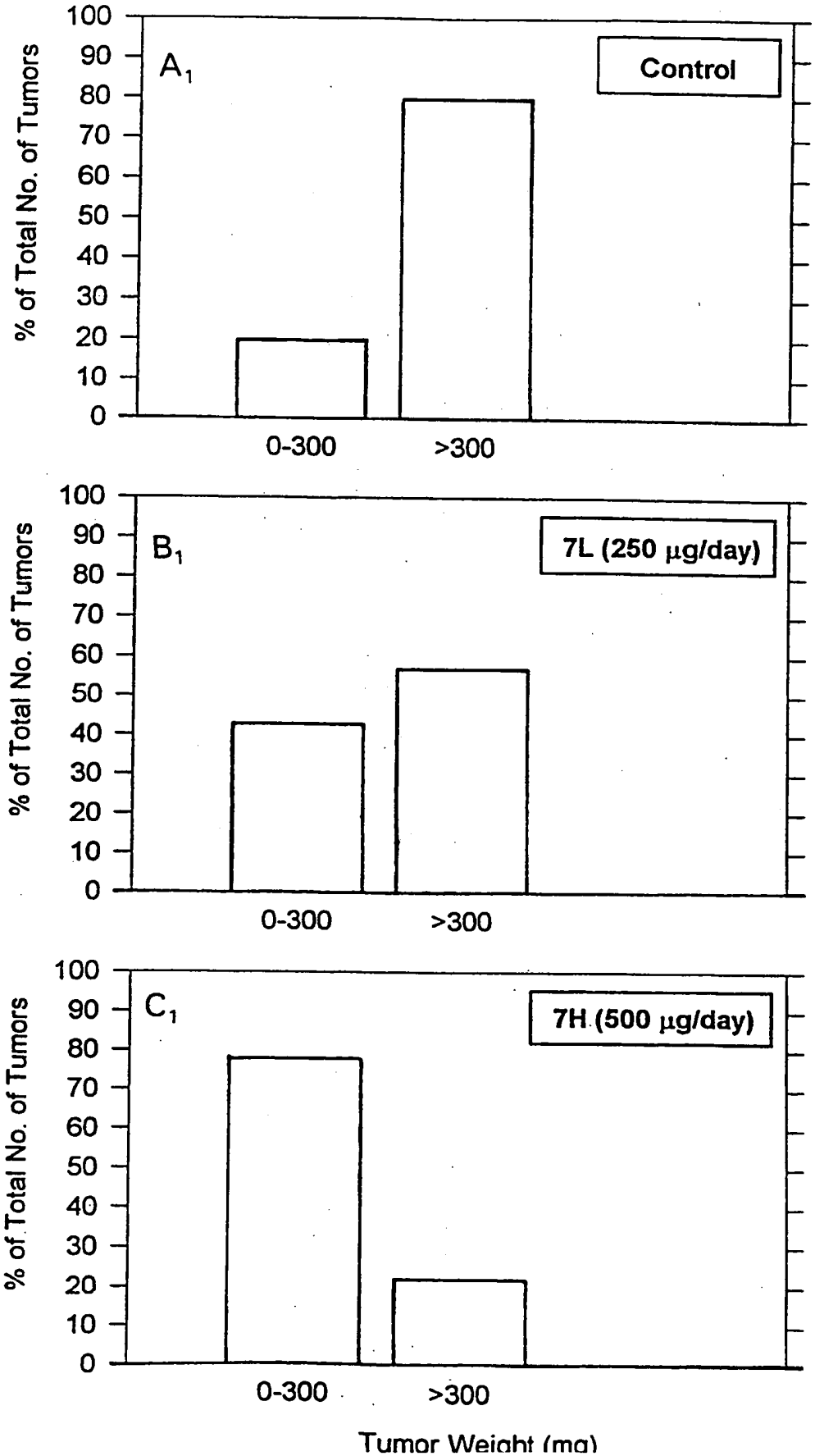


FIG. 15 Tumor volume over time: Control vs. 7H

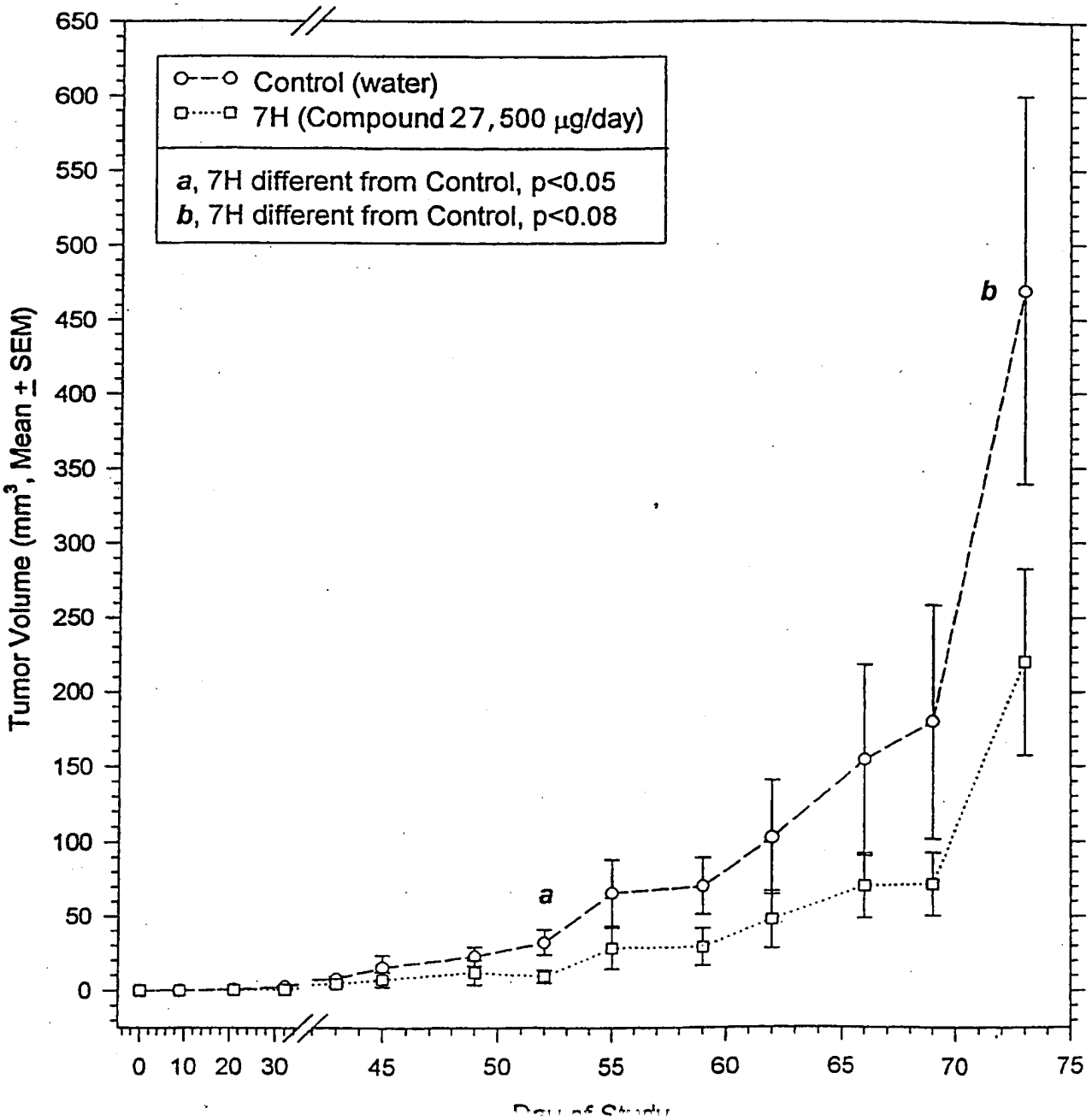


FIG. 16 Tumor volume over time: Control vs. 7L

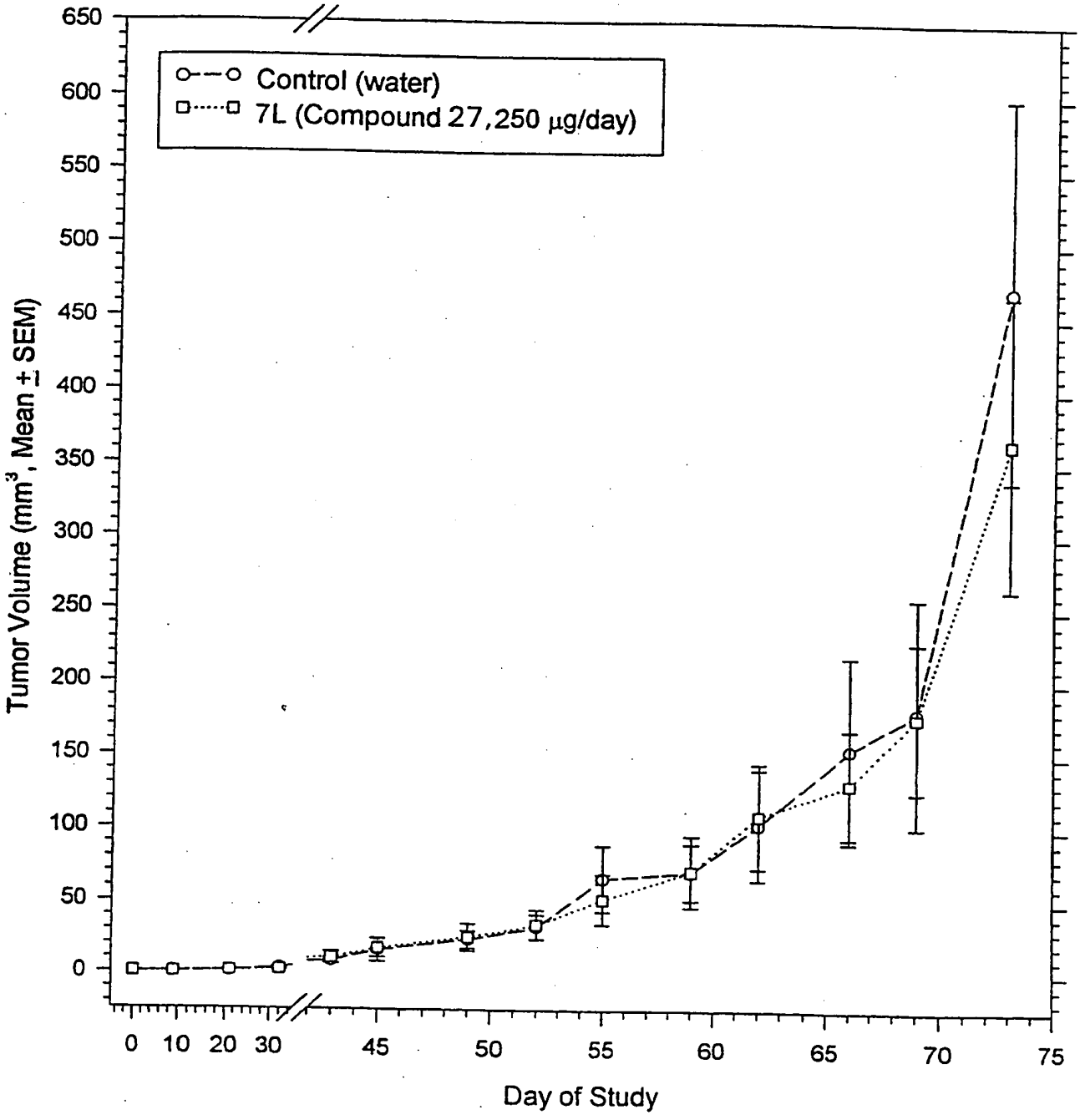
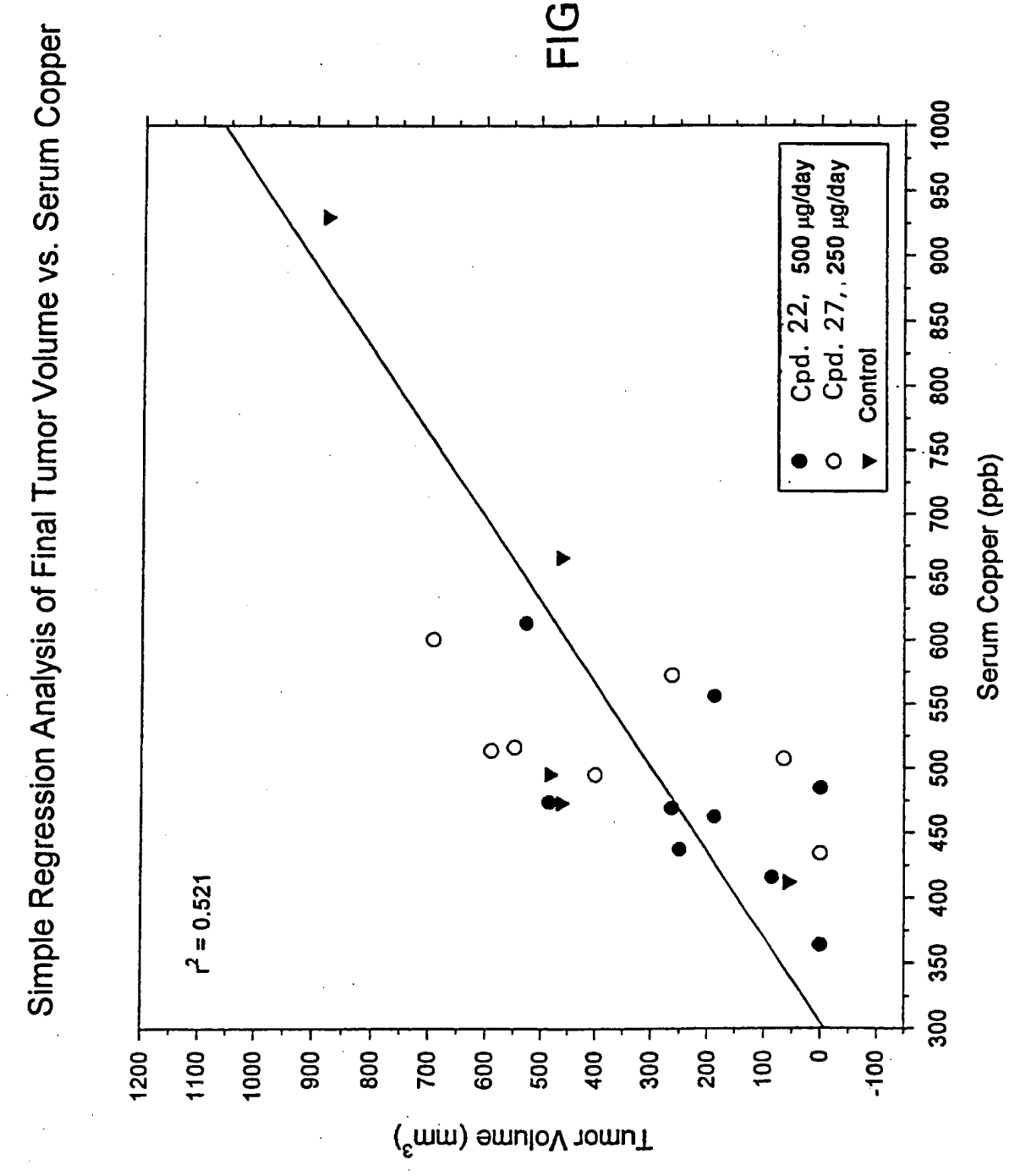


FIG. 17


Simple Regression Analysis of Final Tumor Volume vs. Serum Copper
r^2 = 0.521
Cpd. 22, 500 µg/day
Cpd. 27,, 250 µg/day
Control
Tumor Volume (mm^3)
Serum Copper (ppb)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000

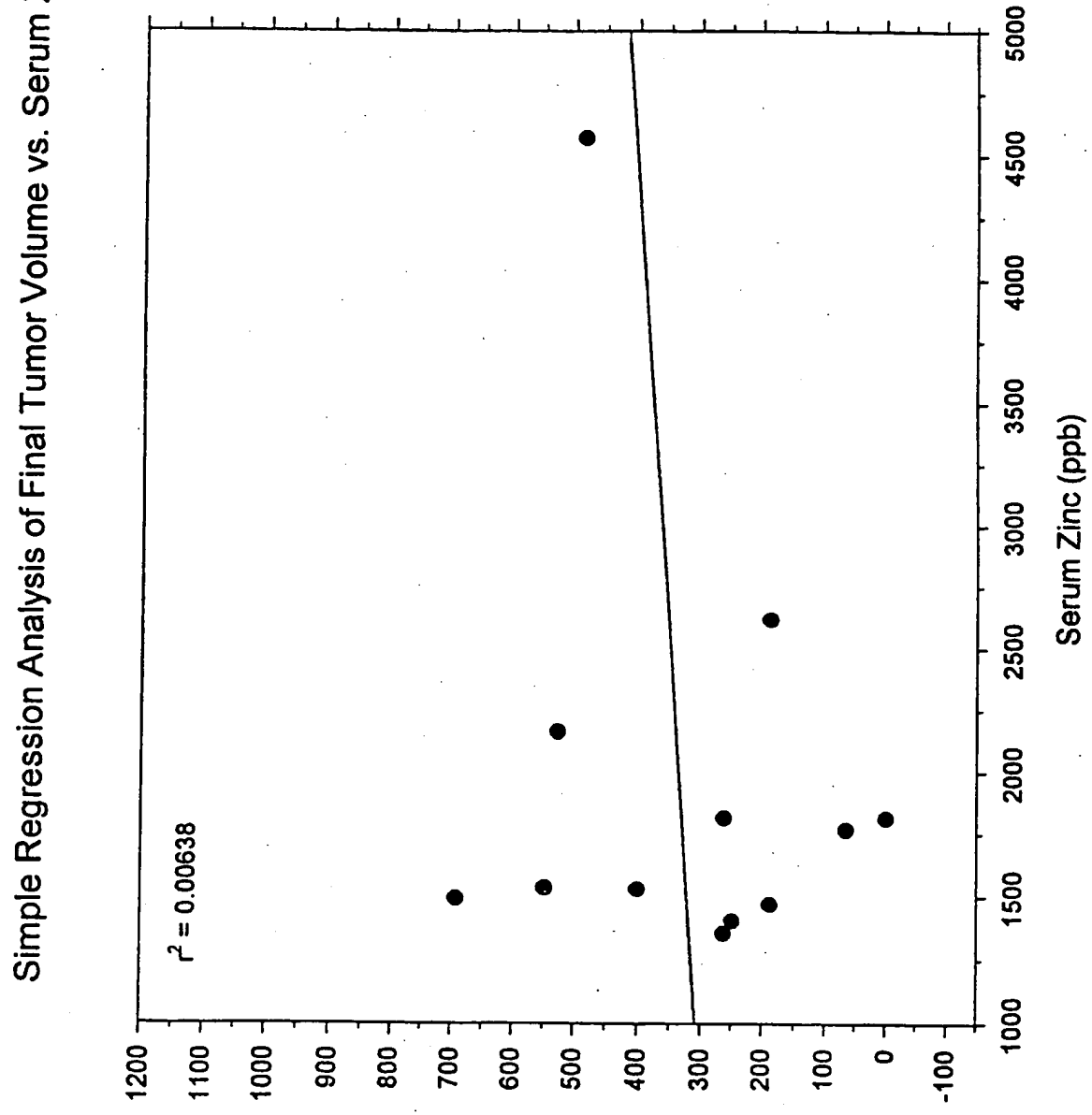

Simple Regression Analysis of Final Tumor Volume vs. Serum Zinc
r² = 0.00638
Tumor Volume (mm³)
Serum Zinc (ppb)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
1000
1500
2000
2500
3000
3500
4000
4500
5000

FIG. 18

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/CA 99/00947

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D257/02 C07C211/29 C07C215/54 A61K31/395 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 125, no. 20, 11 November 1996 (1996-11-11) Columbus, Ohio, US; abstract no. 264195, ZHU S. ET AL.: "Synthesis of novel macrocyclic polyamines with a pendant phenol group and properties and structures of their copper(II) complexes" XP002129113 abstract & INORG. CHEM., vol. 35, no. 20, 1996, pages 5851-5859, --- -/--	1,4-7,9, 10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 2000	10/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chouly, J

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/CA 99/00947

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 126, no. 21, 26 May 1997 (1997-05-26) Columbus, Ohio, US; abstract no. 283202, KOU F. ET AL: "Studies on the formation and decomposition of copper(III) and nickel(III) complexes with macrocyclic ligands containing amide groups" XP002129114 abstract & POLYHEDRON, vol. 16, no. 4, 1997, pages 741-747, ---	1,4-7,9, 10
X	CHEMICAL ABSTRACTS, vol. 127, no. 17, 27 October 1997 (1997-10-27) Columbus, Ohio, US; abstract no. 231033, ZHU S. ET AL.: "Studies of artificial hydrolytic metalloenzymes: the catalytic carboxyester hydrolysis by new macrocyclic polyamine zinc(II) complexes with a phenolic-pendant as novel nucleophile" XP002129115 abstract & POLYHEDRON, vol. 16, no. 19, 1997, pages 3285-3291, ---	1,4-7,9, 10
A	WO 92 16494 A (JOHNSON MATTHEY PLC) 1 October 1992 (1992-10-01) claims ---	1-14
A	CHEMICAL ABSTRACTS, vol. 128, no. 5, 2 February 1998 (1998-02-02) Columbus, Ohio, US; abstract no. 53822, KOU F.P. ET AL.: "Linear free energy relationships in coordination chemistry." XP002129116 abstract & HUAXUE XUEBAO, vol. 55, no. 10, 1997, pages 983-990, -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]onal Application No
PCT/CA 99/00947

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9216494 A	01-10-1992	AU 1372092 A	21-10-1992
		CA 2106068 A	16-09-1992
		FI 933828 A	01-09-1993
		JP 6505728 T	30-06-1994
		NO 933273 A	14-09-1993

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